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INTERNATIONAL UNION OF CHEMISTRY, PURE AND APPLIED

The resolutions adopted at the recent Conference of the International Union of Chemistry, Pure and Applied, by the Committee on Biochemical Nomenclature, are contained in the following extract from the report of the Conference.

III. La Commission de Réforme de la Nomenclature de Chimie biologique adopta des propositions ayant trait aux dénominations les plus générales:

1° Le nom d'un principe immédiat dont la constitution chimique est connue doit être formé d'après les règles de la nomenclature de Chimie organique;

2° Le terme *glucides* désigne le groupe de substances qui comprend les sucres simples réducteurs et les substances qui donnent un ou plusieurs de ces sucres par hydrolyse;

3° Le terme *lipoïdes* est supprimé;

4° Le terme *lipides* désigne le groupe de substances qui comprend les matières grasses et les esters (éthers sels) possédant des propriétés analogues (lécithines, phosphatines, etc.);

5° Le terme *protides* désigne le groupe de substances qui comprend les acides aminés naturels et les substances qui donnent par hydrolyse un ou plusieurs de ces acides aminés.

INTERNATIONAL UNION OF CHEMISTRY.
PURE AND APPLIED

The resolutions adopted by the general meeting of the International Union of Chemistry, Pure and Applied, by the Committee on Unification of Nomenclature, are contained in the following extract from the records of the Conference.

LXXXIV. OBSERVATIONS ON CERTAIN REDUCING AND OXIDISING REACTIONS IN MILK.

By PAUL HAAS and THOMAS GEORGE HILL.

From the Botanical Department of University College.

(*Received July 16th, 1923.*)

I. The reduction of nitrate to nitrite.

In 1904 Kastle and Elvove [1904] first described the reducing action of a substance contained in the sap of the potato tuber upon sodium nitrate in the presence of acetaldehyde and other less potent activators. During the course of an investigation of this and similar reducing reactions of certain plant extracts, comparative experiments were made with cow's milk, since this substance has long been known to exert a reducing action upon methylene blue, both in the presence[1] and in the absence of aldehydes. It was then found that milk also possesses the property of reducing nitrate to nitrite in the presence of acetaldehyde, but it was subsequently ascertained that this observation had been previously recorded by Bach [1911] who had concluded that the action was due to an enzyme which appeared to be present in very small quantity. The experimental evidence here brought forward does not support this view. Later Bach [1912] found that a similar reducing reaction is given by an extract of calves' liver, an observation which was subsequently made anew by Harris and Creighton [1912].

The reduction of sodium nitrate to sodium nitrite by milk is easily demonstrated as follows:

Exp. 1. To 10 cc. of fresh cow's milk, 3 cc. of 4 % sodium nitrate are added, together with two drops of 10 % acetaldehyde; the tube is placed in a thermostat at 50° for ten minutes, then removed, cooled under the tap, and tested for nitrite by the addition of a few drops of Griess-Ilosvay reagent. If nitrite be present a pink colour will appear very quickly and rapidly increase to its maximum intensity, whilst a control in which the nitrate solution is replaced by an equivalent volume of water gives a negative reaction. The intensity of the colour varies with the amount of nitrite present; the normal range is from pale pink to magenta; if in excess, the magenta colour quickly becomes brown.

[1] Schardinger reductase.

Although acetaldehyde is not essential for the process, it is requisite if time is a factor of any importance, since the amount of nitrite produced in its absence is very small even after some hours, and in dealing with milk, with its generally large bacterial flora, the sooner an experiment is completed the better; antiseptics such as toluene, lysol, and chloroform may be employed in prolonged experiments.

Acetaldehyde may be replaced by other aldehydes such as benzaldehyde and cinnamaldehyde; formaldehyde, however, in our experience has a retarding and sometimes an inhibiting action.

The reducing agent is destroyed by boiling, the critical temperature being about 72°, but much depends upon the time of exposure to the high temperature, a fact already commented on by previous authors for enzymes of milk, Schardinger's reductase for instance [Rullmann, 1913, 1914; Seligmann, 1906]. The following typical observations show the importance of the time factor and also that the Schardinger reductase is more sensitive to heat than is the nitrate-reducing agent.

Table I.

Temp. ° C.	Time in minutes	Schardinger reductase	Nitrate reducing agent
70°	17	+	+
72°	5	+	+
	10	+	+ weak
	15	–	+ very weak
75°	15	–	+ very weak
80°	10	–	–

With the object of preparing from milk a clear solution containing the nitrate-reducing agent freed from fat or protein, the milk was coagulated by various means, but after separation of the curd, produced either by excess of acetic or lactic acid or by rennin, the whey no longer reduces nitrate to nitrite. Saturation with ammonium sulphate or the addition of basic lead acetate (6 cc. to 100 cc. of milk[1]) likewise completely removes the nitrate-reducing agent with the proteins, the clear whey then giving negative results. It is this fact which makes the separation of the active principle from milk a matter of considerable difficulty and attempts to do this have so far not met with success.

With regard to the measurement of the activity of the nitrate-reducing agent, the fact that milk contains much protein renders the estimation of small amounts of nitrite a matter of no little difficulty. Many methods were tried and the one finally adopted as giving the most reliable results was as follows. To the volume of milk an equal volume of a saturated solution of ammonium sulphate was added, the clot was filtered off and the whey saturated with ammonium sulphate by the addition of an adequate quantity of the crystalline salt. The mixture was then again filtered through a folded filter paper. The nitrite contained in the filtrate was estimated colorimetrically by means of metaphenylene diamine. On theoretical grounds this procedure

[1] It is well to centrifuge off the fats first.

may be open to question in view of the possible interaction between the ammonium sulphate and the sodium nitrite: it was found, however, that in the time spent in salting out the proteins at the room temperature at which this operation was carried out, the loss of nitrite from this cause was, if any, within the margin of error.

The following illustrates a typical experiment on the reduction of nitrate by milk.

Exp. 2. 450 cc. of milk, contained in a stoppered bottle, were mixed with 30 cc. of 4 % sodium nitrate and 6 cc. of 10 % acetaldehyde. After a thorough shaking, the mixture was left at rest in a thermostat at 45° and only shaken up regularly every 15 minutes when taking a sample for estimation. The experimental results obtained are shown in the form of a curve (Fig. 1).

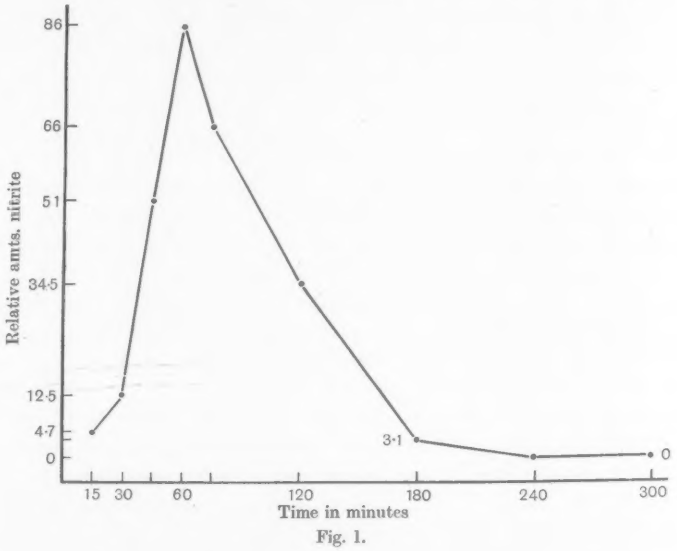

Fig. 1.

This curve is typical for the conditions named, of which the most important is the amount of shaking, since this not only preserves the homogeneity of the fluid but also ensures uniform aeration. The significance of aeration will later be apparent (p. 675).

The curve is peculiar and merits some consideration. If the reduction of the nitrate is a simple enzymic reaction, the curve should ascend until the whole of the nitrate is reduced to nitrite; if the agent is a reducing substance but not an enzyme, the curve should ascend until the whole of this substance is used up. In neither case should there be a fall to zero and it therefore becomes necessary to account for the disappearance of the nitrite. It is unreasonable to suppose that the same agent which reduces the nitrate,

should suddenly break off its action on the nitrate, when the latter is still in excess, and initiate an attack on the nitrite. Even if this were so (provided the agent is enzymic or, if non-enzymic, is in sufficient abundance) the curve should ascend again, since in the experiment there was nitrate sufficient to produce more nitrite. On the assumption that the system is a single agent capable of reducing both nitrate and nitrite, the ultimate result should be that some nitrite should remain just so long as there is an excess of nitrate present, but, the ultimate result should not be zero until all the nitrate has disappeared. The fact that the nitrite ultimately disappears, although excess of nitrate remains, strongly indicates that the mechanism responsible for the production of nitrite has ceased its activity, wherefore it probably is not an enzyme but rather a reducing substance present in limited quantity. Moreover, if the two reductions were really brought about by one and the same substance, whether enzymic or not, the final product of the combined actions alone should be detectable, since the nitrite produced by the first reduction would be in the most favourable condition for immediate further reduction and so would have no opportunity for accumulation to a maximum as is actually the case. These considerations indicate strongly the presence of two active substances; the one reducing nitrate to nitrite, for which we propose the term "atite[1]" the other converting the nitrite into something else. Considerations such as these led to much experimentation which in time yielded evidence which proved the existence of an agent, other than atite (p. 678). Before dealing with this it may be mentioned that on the assumption that the nitrite itself was further reduced, milk was quantitatively examined for ammonia before and after treatment. To do this, increasing quantities of sodium nitrite were added to a series of tubes containing milk and a little acetaldehyde (1 cc. of 10 % aldehyde to 75 cc. of milk) placed in a thermostat at 45°. A small quantity of the mixture from each tube was periodically tested and that tube from which most nitrite had disappeared was selected for estimation as having, presumably, most ammonia. The details of the procedure followed may be omitted since the result showed that the quantity of ammonia present after the destruction of nitrite was no greater than that found in the original milk.

In view of the presence in milk of a reductase capable of reducing methylene blue directly, and also an indirect reductase (Schardinger enzyme) only capable of bringing about this reduction in the presence of formaldehyde [see Lane-Claypon, 1913; Harden and Lane-Claypon, 1912], the relationship between the nitrate reducing agent and the Schardinger reducing enzyme must be inquired into.

That the nitrate reducing agent of the potato is not identical with the Schardinger enzyme is indicated by the following experiment.

Exp. 3. Four tubes are made up and treated as follows:—(1) 1 g. of potato tuber is ground in a mortar under 10 cc. of 4 % sodium nitrate; the

[1] A convenient term since it indicates the conversion of nitr*ate* to nitr*ite*.

mash is placed in a test tube and the temperature raised to 57° in a thermostat; three drops of 10 % acetaldehyde are then added. (2) The same preparation, but the sodium nitrate is replaced by three drops of a 1 % aqueous solution of methylene blue. (3) 5 cc. of milk are mixed with 5 cc. of 4 % sodium nitrate and then as for the first tube. (4) The same preparation as (3), but three drops of methylene blue in place of the sodium nitrate. The four tubes, together with two controls lacking sodium nitrate, are then placed in a thermostat at 57°; numbers (1) and (3) and the controls are removed after two or three minutes and tested for nitrite with Griess-Ilosvay reagent, whilst numbers (2) and (4) are allowed to remain for a time sufficient to give a result. Typical reactions are as follows:

Potato	1. Griess-Ilosvay	+	Control −
	2. Schardinger	−	
Cow's milk	3. Griess-Ilosvay	+	Control −
	4. Schardinger	+	

It may be argued that the negative result with methylene blue is due to the conditions of the experiment: this may be true; but without considering in any detail the botanical aspects of the question, which consideration is reserved for a future occasion, it may be stated that in no instance has success in reducing methylene blue with the sap of various plants been achieved under conditions which invariably gave positive reactions with milk.

II. Oxidation of nitrite to nitrate.

On p. 673 allusion has been made to the significance of shaking, and, in consequence, of aeration. This is well brought out in the following experiments:

Exp. 4. A mixture of 500 cc. milk, 6·6 cc. of 10 % acetaldehyde, and 33·3 cc. of sodium nitrate was placed in a glass container of 1 litre capacity which was throughout the experiment rotated in the thermostat at 45° to ensure continuous agitation and aeration. A precisely similar mixture contained in a vessel of the same capacity was placed in the thermostat but not rotated and only shaken every 15 minutes. Samples from each were periodically taken and the amount of nitrite estimated.

Table II.

Time in minutes	Relative amount of nitrite: Continuously rotated	Relative amount of nitrite: Periodically shaken	Time in minutes	Relative amount of nitrite: Continuously rotated	Relative amount of nitrite: Periodically shaken
15	0	0·04	90	0	0·14
30	0	0·11	120	0	0
45	0	0·22	180	0	0
60	0	0·52	240	0	0
75	0	0·55			

The results indicate that with thorough aeration no measurable amounts of nitrite are detectable, whereas, when the aeration is limited, quantities of

nitrite rising to a maximum and falling off again are produced as in the experiment represented by Fig. 1. A further test was applied by conducting two experiments side by side, the only difference being that the one lacked oxygen.

Exp. 5. 300 cc. of milk together with 20 cc. of 4 % sodium nitrate were placed in a bottle fitted with a cork through which passed a delivery tube reaching to the bottom and an exit tube; the air was exhausted from the bottle and a rapid stream of nitrogen was then bubbled through for about half-an-hour, to wash out the last traces of air. 4 cc. of acetaldehyde were thereupon rapidly added and nitrogen was again bubbled through. The vessel was then closed and placed in the rotating mechanism of the thermostat at 45°. A similar mixture of aerated milk, nitrate and aldehyde was placed in a bottle of suitable capacity and fixed in the rotating mechanism of the same thermostat. Both bottles were thus equally agitated throughout the experiment. Samples from each were periodically removed, without admitting air to the anaerobic tube, and the nitrite estimated, with the following results:

Table III.

Time in minutes	Relative amount of nitrite: Aerobic conditions	Relative amount of nitrite: Anaerobic conditions	Time in minutes	Relative amount of nitrite: Aerobic conditions	Relative amount of nitrite: Anaerobic conditions
30	0	0·024	120	0	1·06
45	0	0·125	180	0	1·18
60	0	0·461	240	0	1·30
75	0	0·80			

The figures in Table III representing the relative amounts of nitrite produced under anaerobic conditions show a gradual falling off, but the duration of the experiment was insufficient to show whether there was a limit to the amount of nitrite which could be produced. For this reason an experiment similar in all respects to the foregoing, with the exception that hydrogen was used instead of nitrogen, was carried out for a longer period. The results are set out in Table IV and Fig. 2.

Table IV.

Time in hours	Relative amount of nitrite	Time in hours	Relative amount of nitrite
1	0·74	5	2·67
2	1·47	7	2·90
3	2·03	8	2·89
4	2·30	9	2·89

From these and like experiments it is clear

(1) That the action of atite is best manifested in the absence of oxygen which element is not a requisite, in that the action of atite is a reductive process.

(2) That the estimations of nitrite obtained in an atite experiment, Fig. 1 for example, represent the balances between the formation and destruction

of nitrite in a given time under the experimental conditions, of which the degree of aeration is of the greatest importance.

(3) That atite is present in limited amount and can only reduce a certain quantity of nitrate.

(4) That the destruction of nitrite, since it is so rapidly effected in a free supply of oxygen, is an oxidative process rather than a reductive process, as was first supposed. This latter conclusion was proved to be correct by the following experiment in which the production of nitrate was established.

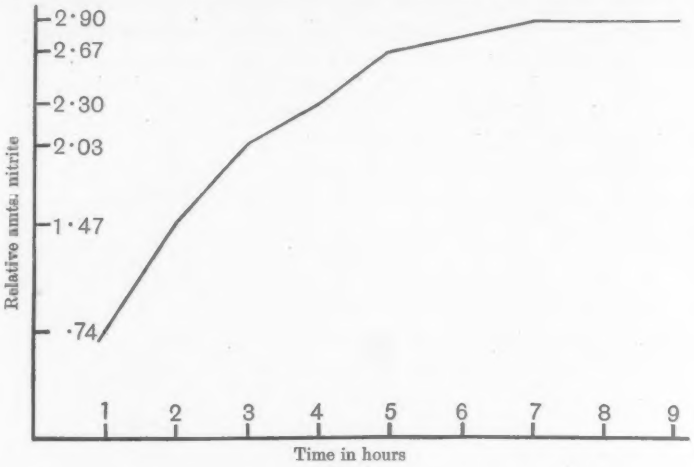

Fig. 2.

Exp. 6. A number of tubes were prepared each containing milk and aldehyde in the proportion of 75 cc. of milk to 1 cc. of 10 % aldehyde; to each tube was added a standard solution of sodium nitrite in serially increasing amounts. The tubes were then rotated in the thermostat at 45° and the contents tested with Griess-Ilosvay reagent at definite intervals of time for nitrite. The accompanying table, from which the lower additions of nitrite have been omitted, sets out the results.

Table V.

Time in minutes	Cc. of standard sodium nitrite added to each tube[1]													
	40	45	50	55	60	65	70	75	80	85	90	95	100	110
30	−	−	+	+	+	+	+	.	.	.	.	.	.	.
60	.	.	−	−	−	−	−	+	+	+	+	+	+	+
90	.	.	.	.	.	.	.	−	+	+	+	+	+	+
120	.	.	.	.	.	.	.	.	−	−	+	+	+	+
150	.	.	.	.	.	.	.	.	.	.	−	+	+	+
210	.	.	.	.	.	.	.	.	.	.	.	−	−	+
390	.	.	.	.	.	.	.	.	.	.	.	.	−	+

[1] In order to prevent undue dilution a stronger solution of nitrite was actually employed for these experiments so that the actual volumes of liquid added were only 1/10 of those given in these columns.

The tube to which 10 cc. of strong nitrite equivalent to 100 cc. of standard sodium nitrite had been added when tested after 6½ hours with Griess-Ilosvay reagent was found to give no reaction, indicating absence of nitrite. In order to ensure absence of traces of nitrite the solution was further warmed with pure nitrate-free urea and hydrochloric acid; it was then tested for nitrate with diphenylamine when a very strong positive reaction was given; this observation has been several times confirmed and the conclusion is therefore reached that the nitrite formed by the action of atite is reconverted back into nitrate. Furthermore this reconversion is effected by some agent contained in the milk, since controls to test the action of atmospheric oxygen and of acetaldehyde upon sodium nitrite in the absence of milk gave negative results. The facts set forth in Table V indicate that this agent, which is responsible for the oxidation of nitrite to nitrate and for which the name of *itate*[1] is proposed, is present in milk in limited amount, the quantity present in 75 cc. of milk being unable to effect the oxidation of 110 cc. of 0·182 % standard nitrite even after 6½ hours. As has been seen, it is only active in the presence of free oxygen and like atite it is dependent on some accelerator or activator such as acetaldehyde (see p. 679). Like atite it is precipitated with the proteins when milk is coagulated and is destroyed in five minutes at a temperature of 99°, in fact its thermolability is similar to that of atite, the thermal inactivation point being between 70 and 75° (Table VI).

Table VI.

Temp. °C.	Reaction with Griess-Ilosvay reagent after the stated intervals of time		
	5 minutes	10 minutes	15 minutes
80	+	+	+ } all reactions
75	+	+	+ } very strong
70	+ weak	+ weak	+ weak
65	+ weak	+ weak	+ very weak

After these observations had been made, it was found, on referring to the literature, that Bach [1911] had also noticed in his experiments on milk a tendency for the nitrite to disappear during the course of the reaction; he was however unable to find a reason for this.

It is well known that peroxidase is described as occurring in milk [for literature see Lane-Claypon, 1913]. The question arises whether the oxidation of nitrite to nitrate is not due to the activity of this peroxidase, or, in other words, whether itate and peroxidase are identical. Experimental evidence goes to show that there is undoubtedly a close connection between the two: invariably has it been observed that the disappearance of the itate activity is coincident with the disappearance of the positive guaiacum reaction and *vice versa*, and conditions which destroy the one will destroy the other. If then itate is identical with milk peroxidase it would at any rate appear doubtful whether it is enzymic in nature, since it is used up in the course of its activity.

[1] Since it converts nitr*ite* into nitr*ate*.

ERRATA

Vol. 17 p. 678, lines 1–2, *for* "standard sodium nitrite"
read "0·0182 % sodium nitrite"
line 15, *for* "110 cc. of 0·182 % standard nitrite"
read "11 cc. of 0·182 % sodium nitrite"

Furthermore, since it was observed that milk which gives a positive reaction with guaiacum and hydrogen peroxide is unable to oxidise nitrite in the absence of acetaldehyde, even if oxygen be present, a comparative experiment was set up using the strong direct oxidase of the potato. But in this case also it was found that no oxidising action was exerted upon added nitrite.

Also it has been found that neither the oxidase of the potato tuber nor the peroxidase of horse-radish is destroyed in the presence of acetaldehyde or of nitrite, whilst the peroxidase reaction of milk is so destroyed under the same experimental conditions. From these facts it is concluded that the so-called peroxidase of milk at any rate differs materially in its properties both from a typical peroxidase and from a typical oxidase of plant origin.

III. The action of acetaldehyde.

Acetaldehyde, or some comparable substance, is either requisite, or is necessary as an accelerator, for the activity of atite, itate and Schardinger reductase. It is, however, peculiar in the fact that it will destroy atite, itate and Schardinger enzyme in the presence of oxygen, which leads to the paradox that the same substance which activates also destroys. The following experiment illustrates this.

Exp. 7. Tubes of milk with added acetaldehyde and sodium nitrite in the proportion of 1 cc. of 10 % aldehyde and 4 cc. of a standard nitrite solution (of ten times the usual strength) to 75 cc. milk were rotated in the thermostat at 45°. After 25 minutes they were removed and tested:

Table VII.

Mixture	Guaiacum	Griess	Methylene blue
Milk alone	+	.	+
„ and aldehyde	−	.	−
„ „ nitrite	+	+	+
„ „ nitrite and aldehyde	−	−	−

The results show that heat alone does not destroy the active principles, that aldehyde destroys both the oxidising itate and the reducing Schardinger, and that itate without aldehyde cannot oxidise the nitrite in the given time.

This dual action of acetaldehyde offers a possible explanation of an observation many times made that itate can in a given period oxidise a definite amount of sodium nitrite in the presence of acetaldehyde; but if the nitrite be presented in two portions, the sum of which is the same as the original amount, oxidation is never completed.

Exp. 8. Four tubes were half filled with 75 cc. milk, 1 cc. acetaldehyde, and 6, 7, 8 and 9 cc. standard sodium nitrite respectively. Rotated in thermostat at 45° and tested after one hour:

Table VIII.

	Amount of nitrite added			
Reagent	6	7	8	9
Griess-Ilosvay	−	−	−	−
Guaiacum	−	−	−	−
Diphenylamine	+	+	+	+

To the first three tubes there were then added 3, 2 and 1 cc. of sodium nitrite, *i.e.* sufficient to bring the total amount of nitrite to 9 cc. which amount was completely oxidised in one hour. Even after the lapse of $2\frac{1}{2}$ hours the tubes gave strong positive reactions for nitrite showing that the preliminary heating with aldehyde had destroyed the itate which should have been capable of oxidising the further added quantities of nitrite.

Similarly atite is destroyed by its accelerator acetaldehyde, a phenomenon illustrated in the following experiment.

Exp. 9. 150 cc. of milk with 2 cc. of 10 % aldehyde were rotated in the thermostat at 45° and periodically tested. After 15 minutes, the milk no longer gave a positive reaction with Guaiacum tincture and therefore presumably the itate was destroyed. The milk, which still contained acetaldehyde, was then divided into two equal portions, to one (A) were added 3 cc. of standard nitrite (strong) and to the other (B) 5 cc. of 4 % nitrate. A control (C) containing 75 cc. of milk, 1 cc. aldehyde and 3 cc. nitrite was also put up. The three tubes were rotated in the thermostat at 45° and periodically examined:

Table IX.

Time in minutes	Test	(A) Added nitrite	(B) Added nitrate	(C) Control
15	Griess	+	–	–
	Guaiacum	–	–	–
45	Griess	+	–	.
	Guaiacum	–	–	.
120	Griess	+	–	.
	Guaiacum	–	–	.

Since the control (C) by its negative reaction with Griess showed that the itate was active, the failure to destroy the added nitrite in (A) must be attributed to the disappearance of the itate, it having been inactivated by the preliminary treatment with aldehyde. Similarly the failure to produce nitrite in (B) must be due to the destruction of atite since, as is shown by (A), the preliminary heating with aldehyde had destroyed the itate. Otherwise expressed there are only two ways in which the absence of nitrite in (B) can be accounted for; one of these is the inactivation of atite and the other the excessive activity of itate in oxidising nitrite to nitrate; this latter alternative is however excluded by (A), wherefore it must be concluded that the atite had been destroyed.

It has been stated above that oxygen is essential for this destruction to take place; to show this the following experiment may be quoted.

Exp. 10. The oxygen was replaced by nitrogen in a mixture of 225 cc. milk and 3 cc. of acetaldehyde and rotated, under anaerobic conditions, in the thermostat at 45°. After $2\frac{3}{4}$ hours, 15 cc. of 4 % sodium nitrate were added, precautions being taken to exclude air, and the rotation continued. After the lapse of one hour the mixture was titrated for nitrite and gave the value 0·677; at the end of the second hour, the titration value was 1·42.

From this it follows that neither prolonged heating, nor the presence of aldehyde in the absence of oxygen impairs the activity of atite. After the titration was made the remainder of the milk was well aerated, replaced in the thermostat and tested with Griess-Ilosvay reagent after the lapse of 15 minutes. A negative reaction was obtained showing likewise that neither prolonged heating nor the presence of aldehyde in the absence of oxygen impairs the activity of itate.

By similar experimentation, details of which it is unnecessary to give, it was found that Schardinger reductase is destroyed by acetaldehyde in the presence of oxygen.

Summarising the evidence relating to the action of acetaldehyde; whilst acetaldehyde is an accelerator, it also in presence of oxygen destroys atite, Schardinger reductase, and itate, that is both reducing and oxidising systems. It is difficult to understand the function of the oxygen in the destruction of substances of so diametrically opposed properties.

IV. The nature of atite and itate.

With respect to atite, Bach considers it to be an enzyme; Kastle and Elvove suggest it might be an oxidisable substance; and Harris and Creighton consider it to be an enzyme and term it reductase.

Any such views as may have been held with regard to atite by earlier authors do not apply to itate since this substance has not been previously described by other workers, although the fact of the disappearance of nitrite is mentioned by Bach. In deciding whether these substances are enzymes or oxidisable and reducible substances respectively, much depends on the relative importance attached to the characters of thermolability and of indestructibility in the normal reaction. For us the latter would appear the more important, and from the experimental evidence brought forward it seems that the most reasonable explanation of the nature of atite and itate is that they are respectively oxygen accepting and donating substances present in milk in limited amount; limited, in view of the fact that they are only capable of reducing or oxidising definite amounts of nitrate or nitrite respectively. At the same time we have not overlooked the possibility of the toxic effect of the aldehyde in gradually depressing the activity of both atite and itate in such a manner as ultimately to mask what to us appears the most characteristic feature of enzyme action, namely indestructibility in a normal reaction. The possibility of atite and itate being the oxidised and reduced forms respectively of one and the same substance, comparable with glutathione, is excluded for the reason that the amount of nitrite which can be oxidised by itate is very much greater than the amount of nitrite which the atite can produce in the course of a normal experiment.

SUMMARY.

1. Milk contains an oxidisable substance which has the power under certain conditions of reducing nitrate to nitrite. Although possessing one of the characteristics of enzymes, namely thermolability, the evidence indicates that it is not a true enzyme, in that it is destroyed in the process of reduction. It is precipitated by the methods commonly employed in coagulating milk. The name of *atite* is proposed for this substance pending more information regarding its nature.

2. Milk also contains a substance capable under certain conditions of effecting the oxidation of nitrite to nitrate. It is active only in the presence of oxygen and under the most favourable conditions will oxidise the nitrite formed by atite as quickly as it is formed, so that under the requisite conditions of the experiment the presence of neither of these two active bodies is discernible. This substance is termed *itate*, and the evidence points to the fact that it is not a true enzyme, in that it is destroyed in the course of its oxidative activity. Its characters resemble those of atite as regards thermolability and precipitation.

3. For both atite and itate an accelerator is required, aldehydes, especially acetaldehyde, being the most potent. The action of acetaldehyde is peculiar in that in the presence of oxygen it destroys atite, itate, and also Schardinger enzyme.

4. The reducing substance known as atite is distinct from the reducing enzyme of Schardinger.

5. Since the disappearance of the guaiacum-peroxide reaction of milk is coincident with the destruction of itate the latter is possibly identical with "peroxidase"; since moreover the conditions which bring about the inactivation of milk "peroxidase" do not similarly affect either the peroxidase of horse-radish or the oxidase of the potato it appears that milk peroxidase differs fundamentally in properties from plant peroxidases.

6. No direct physiological significance is attached to the presence of these two active principles, atite and itate; indeed it is difficult to rationalise such a circulation, nitrate→nitrite→nitrate, in a secretion. To what extent they are present in the milk of mammals generally has not been ascertained.

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LXXXV. STUDIES ON CARBOHYDRATE METABOLISM.

II. ON THE PREPARATION OF AN ANTI-DIABETIC HORMONE FROM YEAST. PART I.

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The importance of phosphorus in the metabolism of carbohydrate is well known. Since insulin relieves the symptoms of diabetes, and carbohydrate-phosphorus compounds are formed by the action of yeast, it seemed possible that a substance similar to insulin might be obtained from this source. It has been shown [Winter and Smith, 1923, 1] that it is possible to extract from yeast a substance which has a similar effect to insulin in causing a lowering of the blood sugar of normal animals. Though this extract, like crude insulin, contains organic phosphorus, Dudley [1923] has shown that insulin purified by the picrate method contains no phosphorus, and that the phosphorus in the crude product has no significance in this connection. A beneficial effect was observed when the extract of yeast was injected into persons suffering from diabetes mellitus [Winter and Smith, 1923, 2]. Later Collip [1923, 1] obtained an anti-diabetic substance from a variety of vegetable sources, but he apparently found that yeast was not a satisfactory source, for he reported twenty failures before an active extract was obtained. Recently Funk and Corbitt [1923] have met with similar variation when yeast was used. We soon found that different samples of yeast gave extracts which differed widely in their activity, some being quite inactive.

In a private communication from Dr H. H. Dale we were informed that from experiments performed in his laboratory no confirmation of our results could be obtained; as much as 400 mg. of the extract was injected into a rabbit without any lowering of the blood sugar being obtained.

The following is a description of some experiments which we have undertaken to determine the conditions under which an anti-diabetic substance could be obtained from yeast. Collip [1923, 1] has suggested the name *glucokinin* for anti-diabetic hormones obtained from sources other than pancreas, and this name will be adopted in the following account.

Experimental.

The method employed for the extraction of the yeast was essentially that devised by Collip [1922] for the preparation of insulin from pancreas, and modified by Dudley [1923].

In our first experiments 2 kilos. of fresh baker's yeast were mixed with an equal volume of 95 % alcohol, and ground up with sand in a mortar. The extraction was allowed to continue at room temperature for two hours. The mixture was then filtered through a coarse pleated paper. Filtration was readily effected by this means. The remaining steps in the process are the same as in the pancreatic preparation, but unlike the extraction from pancreas, there is no difficulty caused by the separation of fat when the concentration of the alcoholic extract is nearly complete. Light petroleum was rarely necessary. The final product is a hygroscopic white powder readily soluble in water. For injection into animals a known weight of this was dissolved in normal saline. The injections were given subcutaneously, rabbits and rats being used as experimental animals. Food was withheld for 24 hours previous to injection. The blood sugar estimations were carried out by Bang's old method [1913].

The local reaction caused by injection of this extract was much less than in the case of insulin. This difference was particularly noticeable when cases of diabetes in man were being treated. The amount of extract necessary to lower the blood sugar to a similar level was much greater than that required when insulin was used. The effect of glucokinin would appear however to be more prolonged (see Fig. 1, p. 692). The action on the blood sugar may be seen from the following example.

Rabbit 2·2 kilos.

Time		Blood sugar %
10.00	... ...	·14
10.10	injection of glucokinin ·06 mg./g. body weight	
11.30	... ...	·10
2.20	... ...	·06
4.30	... ...	·07
6.30	... ...	·09

Though the weight of extract from a given quantity of yeast varied, the same standard dose was always given to each animal (0·06 mg. per g. body weight).

When it was found that certain samples of yeast gave little activity it seemed possible that the properties of glucokinin might be slightly different from those of insulin, and that some modification in the alcoholic process might be necessary. Small samples of the same yeast were taken and extracted with different strengths of alcohol, and in the ensuing stages precipitation was carried out in 70 or 80 % alcohol. No appreciable difference in the activity was observed. The acidity derived from the yeast would appear to be sufficient to ensure the stability of the active principle, assuming that

its properties are similar to those of insulin, since no advantage was derived from the addition of acetate buffer, p_H 4·5, during concentration of the filtrate. Insulin is fairly stable in acid solution but is rapidly destroyed by alkali (ten minutes in $N/10$ sodium carbonate at 100°) [Dudley, 1923].

It seemed possible, however, that glucokinin might be present in the cells as a precursor from which it could be liberated by the action of alkali. Yeast which yielded very little active principle by the ordinary method was extracted with alcohol in alkaline solution, and acidified before concentration. No increase of activity in these cases was observed.

The final precipitation with 80 % alcohol causes a separation of a yellow oily liquid. A small loss occurs at this stage, some of the glucokinin being precipitated. The greater part, however, of the glucokinin remains in solution to be precipitated in 95 % alcohol at − 7°.

The disintegration of the cells by grinding the yeast with sand in a mortar being obviously incomplete, the possibility of splitting the cells by rapid freezing and thawing was tested with a view to increasing the yield from samples of yeast deficient in glucokinin. Through the kindness of Mr W. B. Hardy, we were able to carry out the freezing at a temperature of − 19° in the Low Temperature Station, Cambridge. The yeast was spread out in a thin layer on trays. When thoroughly frozen it was rapidly thawed under warm alcohol in a water bath. Little success resulted, and we were forced to conclude that some factor was lacking in the samples of inactive yeast, and that no more complete extraction would increase the yield of glucokinin. The following is an example.

Rabbit 2·1 kilos.

Time		Blood sugar %
10·00	... ...	·14
10·15	injected (usual process) ...	
12.30	... ...	·12
2.30	... ...	·12
4.30	... ...	·13

Rabbit 2·9 kilos.

Time		Blood sugar %
10·10	... ...	·13
10·30	injected with extract obtained from frozen yeast	
12.45	... ...	·12
2.45	... ...	·11
4.40	... ...	·12

Up to the present we had used only samples of baker's yeast obtained from the same source locally. Since it was possible that the freshness of the yeast might be of importance, samples of baker's yeast were obtained as fresh as possible, and immediately placed in alcohol. Variability was experienced as before, and no definite evidence could be brought to show that the freshness or otherwise of the yeast was responsible for this.

Experiments were next carried out to determine whether brewer's yeast might be more suitable as a source of glucokinin. Samples of yeast were skimmed from beers of different gravity, and placed as soon as possible under alcohol. Some samples were taken at the usual period (end of fermentation), others were collected when the fermentation was proceeding most rapidly. These were necessarily small as considerable quantities of the fermenting fluid had to be filtered before the necessary quantity of yeast could be collected.

Both top and bottom yeasts were also tested. The activity was usually poor, and in the case of a sample of bottom yeast the blood sugar rose. The following are some results obtained from brewer's yeast.

Rabbit 2·5 kilos.

Time		Blood sugar %
10.15		·15
10.30	injected with extract of top yeast	
12.20		·11
2.30		·12
5.00		·14

Rabbit 2·3 kilos.

Time		Blood sugar %
11.00		·13
11.15	extract of bottom yeast	
2.00		·17
5.00		·16
7.00		·13

Rabbit 1·6 kilos.

Time		Blood sugar %
10.00		·13
10.15	extract of yeast collected at height of fermentation	
12.30		·12
2.00		·12
4.00		·12

It was found that when the final precipitation was carried out (in 95 % alcohol at − 7°) the greater part of the precipitate usually settled overnight. The remainder being finely divided took some days to settle. These two fractions were roughly separated. No variation in the activity occurred as will be seen from the following figures.

Rabbit 2·2 kilos.

Time		Blood sugar %
10.15		·14
10.30	first fraction injected	
11.30		·13
2.00		·11
3.30		·13

Rabbit 2·3 kilos.

Time		Blood sugar %
10.45		·13
11.00	second fraction given	
12.30		·13
2.30		·11
5.00		·14

A comparison was next made between the amounts of glucokinin obtainable from samples of the same brand of commercial yeast obtained on different dates. These were treated in the same way, and gave such very divergent results as may be seen from the following figures.

Rabbit 2·7 kilos.

Time		Blood sugar %
11.45		·11
12.15	injected	
2.00		·10
3.30		·08
5.00		·06
6.00		·05
7.00		·05
7.30	convulsions occurred	
7.45	40 cc. 10 % glucose injected	
8.15	appeared normal, eating	
9.00		·09

Rabbit 2 kilos.

Time		Blood sugar %
10.00		·13
10.30	injected	
12.30		·12
2.30		·10
5.15		·13

The batch which caused the convulsions in the above experiment was used in treating hospital cases.

It seemed possible that the activity of poor samples of yeast might be renewed by suitable treatment. It was also possible that a considerable

quantity of the active principle might reach the fermenting liquor as an exudate when the yeast was grown in a suitable medium. Fermentation experiments were carried out using glucose or fructose. Phosphate was added in some experiments in order to increase the rate of fermentation, for it was not impossible that the presence of glucokinin might be associated with the rapid metabolism of carbohydrate which was taking place. The reaction was stopped after different periods by the addition of alcohol. The mixture was then filtered, and the filtrate was made up to 80 % alcohol. The process was completed in the usual manner. Negative results were obtained in every case, confirming the experiment shown above when the yeast had been skimmed at the height of fermentation. We concluded therefore that fermentation was not necessarily associated with the production of increased amounts of glucokinin. Cultivation of the yeast was then attempted under conditions in which the growth and fermentation would be slower. It seemed possible that the presence of glucokinin might be associated with a certain nutritional state of the yeast cell, such as its content of glycogen. The unusual course was adopted of mixing the yeast with potatoes as a source of nutrition; it was hoped that by not supplying the yeast with preformed reducing sugar some other process possibly associated with the production of glucokinin might be brought into prominence. In case potatoes might also be a source of glucokinin, uncooked potatoes were extracted with alcohol and the usual white powder obtained. No effect was observed when this was injected into a normal rabbit. For the cultivation of the yeast the potatoes were boiled, mashed, cooled and mixed with water before the addition of the yeast. In these experiments as little water as possible was added in order to economise alcohol, but sufficient to make the mixture liquid.

Experiment. 500 g. of yeast were added to 500 g. of potatoes in 700 cc. water and well mixed. The mixture was placed in an incubator at 26° and allowed to remain 24 hours. A similar experiment was carried out with the addition of 20 g. di-sodium hydrogen phosphate. At the end of the period of incubation the action was stopped by the addition of alcohol and the process completed as before. The products obtained were tested by injection into rabbits with the following results.

Rabbit 3 kilos.

Time		Blood sugar %
10.15		·13
10.30	injected (+phosphate)	
12.30		·09
2.30		·10
3.45		·12
5.30		·14

Rabbit 2·2 kilos.

Time		Blood sugar %
10.20		·14
10.45	injected (no phosphate)	
12.45		·13
2.40		·12
4.00		·14
6·00		·13

The original yeast as received was almost inactive. The result of similar experiments has shown that the addition of phosphate increased the yield of glucokinin. A sample of French yeast was divided into two parts. The first

was treated by the ordinary method, the other was cultivated with potatoes in the presence of phosphate. The extracts had the following activity.

Rabbit 1·7 kilos.

Time		Blood sugar %
10.00	... ...	·14
10.15	injected (+phosphate)	
11.00	... ...	·10
12.20	... ...	·06
12.30	convulsed	
12.40	... ...	·06
1.00 / 1.15	40 cc. 10 % glucose injected animal normal and eating	
2.00	... ...	·09

Rabbit 1·9 kilos.

Time		Blood sugar %
10.15	... ...	·14
10.30	injected (original yeast)	
12.00	... ...	·13
2.00	... ...	·11
4.30	... ...	·13

The varying effect on different animals is shown in the following experiment in which the sample which convulsed the animal in the foregoing experiment was tested on another rabbit. Only a moderate fall in the blood sugar occurred.

Rabbit 2·1 killos.

Time		Blood sugar %
9.45	... ...	·13
10.00	injected	
11.30	... ...	·10
12.30	... ...	·09
3.00	... ...	·10
5.00	... ...	·13

These two experiments again emphasise the difference of effect which may occur when the same dose is given to animals of the same species, and this must always be a difficulty when tests of substances depend on their action on different animals.

In the above experiments very little grinding of the yeast was possible owing to the admixture with potatoes. It is probable that only very imperfect extraction of the cells by the alcohol took place, as it is well known that substances are extracted from cells only with great difficulty unless the cell membrane is thoroughly disintegrated. The active principle obtained in the above experiments may have been mainly derived from that which had already passed from the cells into the surrounding liquor during fermentation. Bacterial action cannot, of course, be excluded, but the fact that the temperature of incubation was 26° and that incubation was only continued for one day renders it likely that the glucokinin arose from the dominant organism, that is the yeast. Experiments were then carried out to determine whether rupture of the cell membrane would increase the yield of glucokinin. The yeast was mixed with potatoes as before, with and without phosphate. The mixture was then incubated at 26° for one day, and afterwards placed in the cold room at − 19° until thoroughly frozen. It was rapidly thawed under alcohol as before. No increase of activity resulted. The above series of experiments tended to show that the presence of glucokinin was not associated with ordinary growth or fermentation. Similar experiments in which the

mixture was maintained at different temperatures, such as 10° and 15°, gave negative results.

Yeast was allowed to autolyse at 45° for two days. The liquid was then treated in the usual manner. There was no evidence of the presence of glucokinin as a result of this variation. This was to be expected since insulin is destroyed by trypsin and pepsin [Dudley, 1923]. Although glucokinin may not be identical with insulin, it is reasonable to expect that its behaviour towards proteolytic enzymes would be similar. Some of the same yeast was dried in a current of warm air. It was then extracted with alcohol. The product obtained was without action on the blood sugar of a rabbit.

It was possible that rapid aeration might influence the formation of glucokinin. Yeast was mixed with an equal weight of potatoes to which phosphate was added. Enough water was then added to make the mass sufficiently liquid for aeration. A rapid current of air was drawn through the liquid overnight, the liquid being maintained at 26°. A sample of the same yeast was treated in a similar manner but without aeration. The results obtained from the two samples were as follows.

Rabbit 2·1 kilos.		Rabbit 1·8 kilos.	
Time	Blood sugar %	Time	Blood sugar %
10.20	·14	10.30	·13
10.45 injected (without aeration)		11.00 injected (with aeration)	
12.30	·10	12.45	·13
2.00	·10	2.15	·12
4.30	·13	4.45	·13

Since the original yeast had a small content of glucokinin which was increased by incubation of the yeast with potatoes in the presence of phosphate, it might be concluded that the effect of aeration is to destroy any glucokinin which may originally be present. The destruction may be caused by oxidation, or it may be that the metabolism of yeast in the presence of excess of oxygen is altered, and carried out on different lines, which do not necessitate the formation of glucokinin. Since the precise conditions under which glucokinin is formed by the cells, and the effect of glucokinin on carbohydrate metabolism is unknown, it is impossible to come to any conclusion on this point at the present time. The result may also be explained on the basis that the responsible organism was not a yeast but some other type (possibly anaerobic) which was suppressed by aeration.

The foregoing experiments on the effect of fermentation, mixing with potatoes, temperature, and drying, were all made with yeast from the same distillery, which if extracted without additional treatment gave only small amounts of glucokinin. The results showed that the yield could not be increased to a marked extent: the only factor which appeared to have any influence was the presence or absence of phosphates, as seen in the cultivation experiments with potatoes. When phosphate was added the extract was sufficiently potent to cause convulsions in a rabbit. The similar treatment without phosphates gave no increase of activity. This fact merely serves to

emphasise the importance of phosphate in questions connected with carbohydrate metabolism, but the mechanism at work in the increased production of glucokinin is unknown. Harden and Young [1908] showed that the rate of alcoholic fermentation was increased by the addition of phosphate due to increased formation of hexosephosphoric acid being possible. Since rapid fermentation had no effect on the yield of glucokinin, the increased formation of hexosephosphate would appear to be unconnected with the formation of the active principle. It is possible that the effect of phosphates may not be the same on different yeasts as regards their glucokinin content, and that the effect may vary with other cultural conditions.

It seemed probable that samples of commercial yeast from different sources might vary in their content of glucokinin. Samples were obtained from different distilleries and tested. Further samples were obtained at intervals, and it was found that in most cases the yield of glucokinin was small. but that in one case there was constant activity, which however was not so great as that of the yeast used in our first experiments. It was possible that there might be some factor of nutrition which was only present in the process employed in this distillery, or that there might be present a small amount of a certain strain of yeast which was almost the sole origin of the glucokinin.

Experiments have been carried out on these lines, with the result that a strain of yeast has been isolated, which gives a yield of glucokinin greater than that ever obtained previously. This property seems to be maintained, as successive batches have continued to prove active.

An investigation into possible means of increasing the yield of glucokinin from such yeast, and on the factors which lead to its formation, is in progress. The results will be given in Part II of this paper.

Discussion.

The fact that an extract of yeast causes a fall in the blood sugar of normal animals affords no proof that it contains an anti-diabetic hormone. In those experiments in which only a small fall of blood sugar occurred, it would be still more difficult to maintain that it was due to a hormone of this type, and not to the large amount of other substances which are also present in the extract, such as breakdown products of proteins. For this reason we have refrained from injecting large quantities of yeast extract, and from continuing the blood sugar determinations for longer than nine hours after the injection. The effect of starvation might possibly affect the condition of the animal to such a degree that the effect of the foreign substances, other than glucokinin, in the injection might begin to manifest themselves. We have emphasised the delayed action of glucokinin as compared with insulin, but this delay (as measured by the time when convulsions occur) we have never found to be more than eight hours.

In a recent paper Collip [1923, 2] gives some results of injecting an extract of yeast into normal animals. His results appear to be very different from those

which we have obtained. He uses several different processes (among them freezing) in obtaining his extract, but the action on the blood sugar appears to have been extremely delayed. No injection figures are given, but from the fact that one animal received nine injections in the course of twenty hours and another six in fourteen hours, it would appear that very large quantities of extract were administered. During the whole of this time the animal was without food. In the cases in which convulsions are recorded the animals died. It is necessary to emphasise here that in every case in which convulsions have occurred we have found complete recovery of the animal to ensue after injection of glucose. It would seem therefore that the anti-diabetic hormone which Collip claims was present in his extracts must be of a totally different type from the one present in our extracts, or that his extracts were so weak that vast quantities were necessary to obtain an effect. In this case the quantities of other substances present would probably have an injurious effect on the animal after more than a day's starvation with repeated withdrawals of blood. The Schaffer and Hartmann [1920] estimation of blood sugar was used by Collip. This method requires 1 cc. of blood for an estimation. We have always used Bang's old method, because of the small quantity of blood that is required, and the ease of obtaining it on an absorbent paper without serious inconvenience to the animal.

There appears to be little doubt that by using a suitable yeast a genuine anti-diabetic hormone can be obtained. It has been found [Winter and Smith, 1923, 2] that following administration of yeast extracts to diabetic patients, excretion of sugar is stopped, and the ratio copper reducing power to polarimetric value of the blood sugar is altered in the same way as after injections of insulin [Forrest, Smith and Winter, 1923].

MacLeod [1923] has suggested that such extracts may merely stimulate the secretion of insulin by the pancreas when injected into normal animals. The results with diabetic cases [Winter and Smith, 1923, 2] would appear to show that just as beneficial an effect may occur as when pancreatic insulin is used; and that therefore, in our opinion, the evidence is strongly in favour of a true anti-diabetic hormone being obtainable from suitable yeasts.

SUMMARY.

1. It is shown that a substance may be extracted from yeast which has an effect similar to that of insulin on the blood sugar of normal animals.

2. When convulsions occur as the result of the injection of this substance, the animals may be recovered by injections of glucose.

3. Samples of yeast obtained from different sources yield varying amounts of glucokinin.

4. By allowing the yeast to ferment under different conditions, it is found that only in the case of an originally active yeast is the glucokinin formation increased under certain cultural conditions.

We wish to express our grateful thanks to Professor F. G. Hopkins for his continued interest in this work, and for providing the large amount of material used in this necessarily expensive research.

Our thanks are due to Messrs D. and D. F. Scanlan for much help during the course of this work, to Dr H. H. Dale for keeping us informed of the progress of the work on insulin which has been carried on in his laboratory, to Messrs Bishop and Brooke for obtaining for us regular supplies of yeast from different sources, and to Messrs H. and G. Simonds, Reading, for giving us every help in the investigation of brewer's yeast.

One of us (W. S.) is indebted to the Scientific and Industrial Research Board, and one (L. B. W.) to the Medical Research Council for grants held during the course of this research.

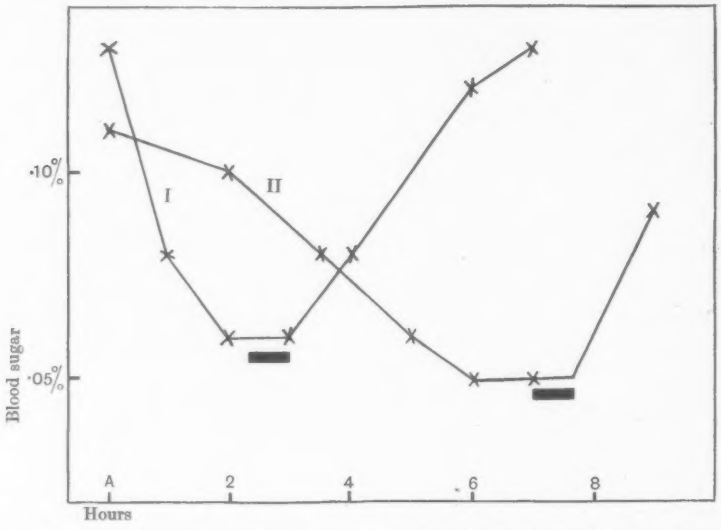

Fig. 1. Curves of blood sugar values of rabbits: I, after insulin (pancreatic); II, after yeast extract. At A the injection was given. The thick lines denote the period during which convulsions occurred. At the end of this period the animals were recovered by injections of glucose. The delayed action of the yeast extract is shown. Time in hours.

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LXXXVI. THE BASIC DISSOCIATION CONSTANT OF VALINE.

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(*Received August 3rd, 1923.*)

A knowledge of the basic dissociation constant of valine was desired by the writer in connection with the methods which he has recently devised [1923] for estimating amino-acids by titrating under conditions governed by K_a and K_b values.

The present determination has been derived from the p_H-neutralisation curve. Known volumes of valine dissolved in water at a given concentration were treated with varying amounts of 0·1*N* HCl, and the resulting p_H values determined by means of the quinhydrone electrode. p_H was calculated from the observed E.M.F. reading by the formula

$$p_H = \frac{c - (\text{E.M.F. obs.})}{\cdot 059},$$

the value of the calomel half cell, c, being determined by reading against solutions of accurately known p_H : 0·1 HCl and buffer solutions of amino-acids plus HCl. All determinations were carried out in a box at 25°.

To obtain the "ideal titration curve," each reading was corrected for the amount of acid necessary to bring the solvent alone (without valine) to the same p_H and the same total volume, in the manner previously described by the writer [1923], and in accordance with the formula

$$i = d - \frac{ge}{100}$$

where i = corrected amount of acid used in titrating to a given p_H;

d = uncorrected amount of acid used in titrating to a given p_H;

g = blank for 100 cc. (no. of cc. of $N/10$ acid necessary to bring solvent alone to the given p_H and to a final volume of 100 cc.);

e = final volume of titrated fluid, after addition of d cc. of HCl.

The value of g corresponding to each p_H reading is based on the formula

$$p_H = -\log\left(\alpha \cdot \frac{g}{0{\cdot}001}\right)$$

and in practice is read from a curve constructed by the writer [1923].

In the table below are shown the results obtained in a typical titration of valine with HCl, and also the manner of introducing the blank correction to obtain the ideal curve.

Titration of 10 cc. of M/20 valine.

(*d*)	(*e*)	(*f*)	(*g*)	(*h*)	(*i*)
N/10 HCl added cc.	Total vol. of liquid after titration ($e = d + 10$) cc.	Corresponding p_H values	Blank for 100 cc. total vol. (read from curve) cc.	Blank for given total vol., e $\left(h = \frac{ge}{100}\right)$ cc.	Corrected vol. of *N*/10 HCl added ($i = d - h$) cc.
0·1	10·1	3·98	—	—	0·1
0·4	10·4	3·38	0·5	0·05	0·35
1·0	11·0	2·93	1	0·1	0·9
1·3	11·3	2·79	2	0·2	1·1
1·6	11·6	2·69	2	0·2	1·4
2·0	12·0	2·58	3	0·4	1·6
2·3	12·3	2·51	4	0·5	1·8
3·0	13·0	2·34	5	0·7	2·3
3·2	13·2	2·29	5	0·7	2·5
3·4	13·4	2·24	6	0·8	2·6
4·0	14·0	2·12	8	1·1	2·9
5·0	15·0	1·96	12	1·8	3·2
6·0	16·0	1·85	15	2·4	3·6
7·0	17·0	1·78	19	3·2	3·8
8·0	18·0	1·69	23	4·1	3·9
9·0	19·0	1·64	25	4·8	4·2
10·0	20·0	1·58	29	5·8	4·2
12·0	22·0	1·51	35	7·7	4·3
15·0	25·0	1·46	40	10	5

Values in columns (*g*) are given to the nearest integer, in columns (*h*) and (*i*) to the first place.

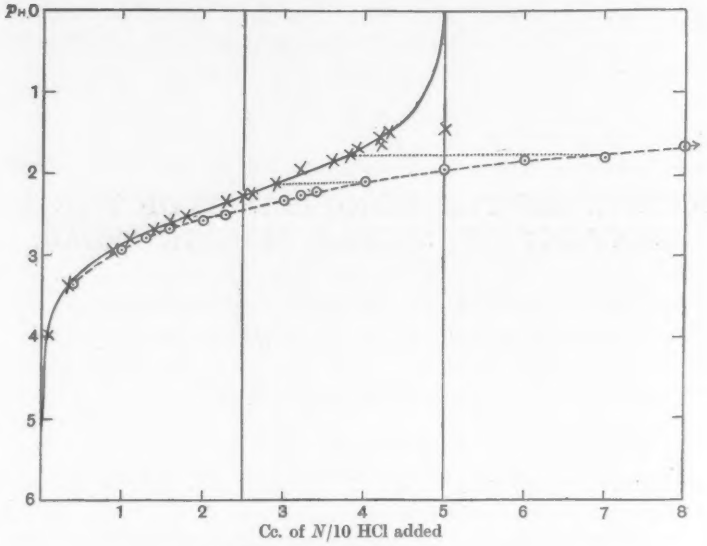
In successive titrations agreement between duplicates could be obtained to within about p_H 0·2.

In the diagram, circled points represent uncorrected readings, crosses the ideal corrected values deduced therefrom. The latter are in very close agreement with the titration curve[1] of a base with $K_b = 2 \times 10^{-12}$, which is represented by the unbroken line calculated from the formula

$$\log \frac{1}{[H^+]} = \log \frac{K_b}{K_w} + \log \frac{(1-\alpha)}{\alpha}.$$

The quinhydrone electrode has not previously been utilised for the determination of a dissociation constant, but it should prove of general applicability when used in conjunction with the above method. The writer has also found that provided it be properly standardised it may be conveniently employed for the accurate determination of amino-groups in amino-acids and generally of both basic and acidic groups.

[1] At the acid end of the titration the ideal corrected values tend to lie around the theoretical curve rather than to be strictly coincident with it, due to the very large magnitude of the blank corrections; a very slight percentage error in reading the correction, *g*, leading to a comparatively large error in the ideal corrected value.



⊙⊙ Uncorrected readings.
× × Readings as corrected for blank.
—— Theoretical curve for $K_b = 2 \times 10^{-12}$.

Summary.

Known amounts of HCl were added to valine and the resulting p_H values were determined by means of the quinhydrone electrode. Correcting for the effect of the solvent by the writer's method the ideal titration curve was deduced and showed a value of $14 - p_{K_b} = 2{\cdot}3$, or $K_b = 2 \times 10^{-12}$ at a dilution of $\frac{M}{20}$, and $t = 25°$.

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LXXXVII. ON THE SIGNIFICANCE OF THE ASH CONTENT OF CERTAIN MARINE ALGAE.

By PAUL HAAS and BARBARA RUSSELL-WELLS.

From the Botanical Department of University College.

(*Received August 4th, 1923.*)

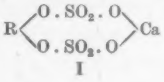
Introductory.

Comparatively little attention has, in the past, been paid to the question whether certain ash constituents occurred as such in the plant or whether they had been produced during the process of incineration; thus while the sulphate or phosphate in an ash may be due to the accumulation within the plant of salts containing these radicles, they may, on the other hand, have been produced during incineration by the oxidation of organically combined sulphur or phosphorus, more especially if the ash has a strongly alkaline reaction, for in such cases the incineration amounts almost to alkali fusion.

It has long been known that many sea-weeds on incineration give ashes containing a relatively high percentage of calcium sulphate, but no particular significance was attached to this fact by earlier workers. By means of micro-chemical tests it should of course be easy to recognise the presence of certain ash constituents and so to establish their pre-existence within the plant before incineration. Such reactions, however, as can be carried out are due to dissolved ions and indicate the presence of salts which it should be possible to remove by dialysis. If such be the case no great significance attaches to their presence as they may merely be mobile products of metabolism or may be part of the plant's saline nutrients. If then a plant, or its aqueous extract still retains a considerable quantity of ash constituents even after exhaustive dialysis there is reason to suspect that such constituents are held in some form of chemical combination which precludes their free movement and consequently renders them incapable of dialysis.

It was found some time ago [Haas, 1921] that *Chondrus crispus* presents such a case, in which the ash is rich in calcium sulphate even after the material has been subjected to prolonged dialysis. Both chemical and micro-chemical analysis revealed the presence of ionised calcium but failed entirely to disclose any sulphate. To account for these facts it became necessary to

assume the existence in the plant of some form of masked sulphate such as an ethereal sulphate of the type represented by the formula



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Chemical evidence for the correctness of this assumption was furnished by the fact that the masked sulphate could be converted into ionised sulphate by hydrolysis.

One of the consequences of this observation has been the recognition within the plant of colloidal electrolytes, a class of substances whose existence had not hitherto been suspected in this connection. The establishment of the presence of ionised calcium attached to a complex colloidal aggregate suggested the possibility for the manifestation of a variety of interesting physical chemical phenomena, of which osmotic pressure and conductivity are the most obvious. By means of the very simplest form of parchment osmometer it has been possible to demonstrate the existence of sufficient osmotic pressure to produce a steady rise in the stem of the thistle funnel. Conductivity measurements were kindly carried out for us by Mr F. C. Harwood in the physical chemistry laboratory of this college, and a preliminary account of some of his results has already appeared elsewhere [1923].

What may be the significance to the plant of the presence of osmotically active colloidal electrolytes in the cell wall is at present a matter of speculation. Whether these substances represent transitory phases in which the plant retains calcium sulphate in chemical combination, to be hydrolysed when occasion arises, it is not as yet possible to say; further evidence will have to be accumulated before it can be stated whether there is any seasonal variation in the occurrence of such compounds.

That the occurrence of ethereal sulphates is a fairly widely distributed phenomenon is shown by the present results in which the occurrence of similar compounds is described in the following Rhodophyceae and Phaeophyceae: *Ceramium rubrum, Delesseria sanguinea, Delesseria alata, Polysiphonia fastigiata, Plumaria elegans, Ascophyllum nodosum* and *Laminaria digitata*.

Since we commenced work on this subject Neuberg and Ohle [1921] have been able to establish by methods similar to our own the existence of the ethereal sulphate grouping in agar, the extract of a species of *Gelidium*. The physical chemistry of this substance has likewise since received attention at the hands of various workers, notably Samec and his collaborators [1921, 1922], and more recently Fairbrother and Mastin [1923].

In view of these facts it would appear necessary to urge the desirability of a more careful examination of the ash constituents of plant material in general with the object of elucidating, if possible, the nature of the combination of these substances in the plant.

Methods.

In the case of *Chondrus* the proof of the existence of an ethereal sulphate depended on the fact that the percentage of sulphate contained in the hydrolysed solution of the hot extract (H.E.) was just twice that contained in the ash, in accordance with the equation:

$$R\begin{matrix}O\,.\,SO_2\,.\,O\\O\,.\,SO_2\,.\,O\end{matrix}\!\!>Ca + 2H_2O = R\begin{matrix}OH\\OH\end{matrix} + CaSO_4 + H_2SO_4$$

from which it is clear that of the two molecular proportions of sulphate here given only the one combined with the calcium would be found in the ash, the other being lost during incineration.

In endeavouring to establish the existence of ethereal sulphate groupings in other material by similar means it has however not always been found possible to establish the exact relation of 2 : 1. The reasons for this discrepancy may be due either to experimental difficulties connected with the incineration or the hydrolysis, or to the inherent properties of the particular type of ethereal sulphate concerned.

A. With regard to difficulties encountered in the hydrolysis it has been found that the reaction is somewhat slowly effected by boiling with hydrochloric acid, due possibly to a tendency towards a reversal of the reaction as noted by Cunningham [1918]. The use of potassium chlorate or other oxidising agent during hydrolysis to assist in the destruction of the organic complex is of course precluded in this connection, since it is essential to ensure that all the sulphate found is due to hydrolysis and that none shall have been produced by the oxidation of organically combined sulphur. After a number of trials however the following conditions were found to effect the complete hydrolysis of such ethereal sulphates, the accuracy being confirmed by simultaneous estimation of total sulphur by Benedict's method.

About 0·75 g. of the substance is placed in a tall form beaker with 60 cc. of water to which 40 cc. of concentrated hydrochloric acid are added. The beaker is then covered with a clock-glass and heated over a sand-bath for six hours, water being added from time to time to replace that lost by evaporation. The resulting dark coloured solution is then filtered from any brown insoluble matter, which is nearly always present, and the filtrate is precipitated with barium chloride.

With regard to the incineration it has been found that the results obtained are very often rather more than half those obtained by hydrolysis. This is notably the case where the ash is strongly alkaline, *e.g. Laminaria* (p. 703), whereas on the other hand in the case of *Chondrus* hot extract, in which the ash is neutral, the ratio comes out exactly 2 : 1.

B. As regards the effect of the type of ethereal sulphate combination upon the ratio it must be borne in mind that the occurrence of an ammonium salt of the type R . O . SO_2 . ONH_4 will at once throw out the ratio, since the

whole of its sulphate will be lost on incineration; such a case has already been described [Russell-Wells, 1922]. Similar remarks apply to the case of a true ethereal sulphate of the type $R_1 . O . SO_2 . O . R_2$ in which R_1 and R_2 are both organic complexes; in the case of such a compound of course the whole of the sulphate radicle should likewise be lost on incineration.

The general method of procedure may now be given, as it is essentially the same for all the cases investigated.

The carefully hand-picked material is extracted with hot water over a water-bath or in an autoclave at 105°; the extract is then filtered over a Buchner funnel, first through calico and then through Chardin filter paper. The filtrate is thereupon placed in a parchment dialyser with a little thymol and the water is replaced morning and evening until it no longer gives any reaction for either sulphate or chloride; the dialysed liquid is evaporated to dryness on a platinum dish and the resulting scales are dried for analysis.

The necessity for thorough dialysis must be emphasised inasmuch as no great significance attaches to sulphate found in a hydrolysed solution unless precautions have been taken to ensure removal of any free sulphate, a consideration which is particularly important in the case of sea-weeds which are normally surrounded by a medium containing sulphates.

With the object of saving time a few analyses were carried out directly upon the weed, without preparing an extract, on the assumption that while adhering sulphate would undoubtedly throw out the ratio, indications of a distinctly higher proportion of sulphate on hydrolysis would be reliable evidence of the presence of ethereal sulphate. Although the method gave valuable indications in some cases it cannot be recommended owing largely to the fact that many weeds give a strongly alkaline ash, which tends to retain some of the sulphuric acid that should be lost on incineration; this is particularly the case with *Laminaria* (p. 703).

The weeds which have been examined for the presence of ethereal sulphate were all selected for their relatively high ash content and the high proportion of sulphate in their ash and it may be safely assumed that this is a reliable criterion for judging of the likelihood of such combinations occurring in any new plant material.

In deciding from the results of an analysis whether a substance contains ethereal sulphate, undue importance should not be attached to the establishment of the exact ratio 2 : 1; the deciding factor should rather be that the dialysed product yields on hydrolysis a relatively high proportion of sulphate and that this proportion exceeds that found in the ash.

Evidence of the occurrence of phosphoric acid in organic combination has also been obtained in the case of *Chondrus*, but here it is of course not possible to rely upon the difference in the amounts found on hydrolysis and incineration since phosphoric acid is not volatile; the evidence is therefore based entirely upon the establishment of phosphate in a dialysed solution after hydrolysis.

Experimental.

Chondrus crispus.

During the dialysis of the hot and cold extracts (H.E. and C.E.) of carrageen it was noticed that these substances, even when free from dialysable salts, drew a very considerable volume of water into the dialyser indicating that they must exert a definite osmotic pressure. To verify this, solutions of pure C.E. and H.E. of the same strength were placed in simple osmometers consisting of thistle funnels with pig's bladder stretched across them. In a short time a rise was observed in each case which was maintained for as long as the tube was left in position, a matter of about a week. A very much greater rise was observed in the case of the C.E. than in that of the H.E.

To obtain some measure of the osmotic pressure exerted by the C.E. in aqueous solution a freezing point determination was made with the following results:

A 1·5 % solution gave $\Delta = 0{\cdot}045°$ which corresponds to an osmotic pressure of 0·54 atmosphere.

Furthermore measurements kindly carried out for us by Mr F. C. Harwood showed the molecular conductivity at the same concentration to be 65·1 and that at infinite dilution to be 110, giving a value for the degree of ionisation of $\alpha = 0{\cdot}59$.

A calculation of the minimum molecular weight of the C.E. from its calcium content of 4 % gives the value 1000, while from the depression of 0·045° the value 620 is obtained, which is in fairly close agreement with value 590 obtained by multiplying the theoretical molecular weight of 1000 by the degree of ionisation 0·59.

The evidence obtained from the conductivity measurements moreover points to a basicity of 2 for the acid radicle combined with the calcium, which lends support to the formula I (p. 697) previously ascribed to this substance in preference to the possible alternative formula II

$$\underset{\text{II}}{\text{R . O . SO}_2\text{ . O—Ca—O . SO}_2\text{ . O . R}}$$

With regard to the question of the occurrence of ethereal phosphate estimations were made on two occasions of the amount of phosphate in solutions of carefully dialysed H.E. when it was found that the amount contained in this material was 0·11 % calculated as P_2O_5. We have however some hesitation in putting forward this figure as the correct value for the amount of phosphoric acid in the material, since we have experienced great difficulty in attempting to hydrolyse quantitatively samples of pure sodium glycerophosphate, a substance of relatively simple constitution when compared with H.E. Similar difficulty in hydrolysing ethereal phosphate has been experienced by Robison [1922].

Ceramium rubrum.

For this weed, as for all others, the ash content varies according to the amount of washing it has received previous to analysis, as may be seen from the following figures obtained from different samples: 11·58, 12·32, 13·05, 13·88, 25·41; it must however be borne in mind that excessive washing is to be avoided owing to the danger of losing some water-soluble constituents other than salts.

In the first instance a direct analysis of the weed was made without extraction; the sample selected was the fourth one quoted above which had been collected in the neighbourhood of Plymouth during the month of May. The following figures were obtained:

	%
Ash in the crude weed	13·88
SO_4 in above ash	15·63
SO_4 in weed by hydrolysis	4·13
SO_4 „ „ incineration	2·17

It was then decided to examine an extract of the weed and for this purpose a sample obtained from Port Erin during the month of April was heated with water in an autoclave for one hour at 110°; after filtering, the residue was heated once more with a fresh quantity of water and the combined extracts were dialysed and evaporated; the dried material, weighing about 10 % of the air dry weed, forms clear light yellow gelatinous scales which swell up considerably in cold water and dissolve completely on standing without the application of heat.

Samples of the dialysed material from different sources gave the following figures on analysis:

	%	%	%
Ash in the extract	9·2	8·35	7·88
SO_4 in the above ash	36·03	37·62	42·76
SO_4 in the extract (by hydrolysis)	8·18	—	8·85
SO_4 „ „ (by incineration)	3·31	3·14	3·37

It will be seen that in this case the ratios of the two sulphate estimations in the first and third cases are 2·47 : 1 and 2·62 : 1 respectively; as explained above this may be due either to the presence of ammonium groups or to the occurrence of a true organic ester; the matter is still under investigation.

To determine whether the substance has any osmotic properties a solution was prepared from 1·2 g. of the extract in 70 cc. of water. Placed in an osmometer there was a rise of 9·3 cm. in seven days thus showing that the substance exerts a distinct osmotic pressure.

Delesseria sanguinea.

In this case the weed, collected near Plymouth in May, was rapidly washed in two or three changes of water and then dried. Analysis of the air dry material gave the following figures:

	%
Ash in the washed weed	16·83
SO_4 in above ash	44·15
SO_4 in the weed (by hydrolysis)	14·47
SO_4 „ „ (by incineration)	7·43

An extract of the above was prepared by heating for two hours in an autoclave at 110°; the mucilaginous solution on evaporation yielded clear gelatinous scales which are readily soluble in water. Analysis of the dialysed material gave the following results:

	%
Ash in the extract	16·01
SO_4 in the above ash	68·39
SO_4 in the extract (by hydrolysis)	23·84
SO_4 „ „ (by incineration)	10·68

Osmotic properties were established by means of the osmometer in which a solution containing 1·5 g. of dialysed extract in 70 cc. of water registered a rise of 3 cm. in 40 hours. These facts together with the ratio of 2·23 : 1 for the two sulphates confirm the presence of ethereal sulphate.

Delesseria alata.

In this case only the weed itself was analysed. The material was kindly collected for us at Port Erin in July by Miss Knight, of Liverpool University. Analysis gave the following results:

	%
Ash in the washed weed	12·24
SO_4 in the above ash	33·84
SO_4 in the weed (by hydrolysis)	9·77
SO_4 „ „ (by incineration)	4·17

Here again the excess of sulphate obtained by hydrolysis over that found on incineration proves the presence of ethereal sulphate.

Polysiphonia fastigiata.

The ash content of this weed again depends very much upon the amount of washing, as is shown by the following figures obtained from the same sample before and after washing:

	%	%
Ash before washing	24·88	28·19
Ash after washing	9·34	9·99

Analysis of an unwashed sample gave the following figures:

	%
Ash in the unwashed weed	24·88
SO_4 „ above ash	24·84
SO_4 „ unwashed weed (by hydrolysis)	11·80
SO_4 „ „ „ (by incineration)	6·18

An extract was made of a sample of weed kindly collected for us near Folkestone in the month of March by Mr Chater of this Department. Heated at 105° for one hour the weed yielded to water about 20 % of its weight, giving a mucilaginous solution which on evaporation left light brown shiny transparent scales.

The substance is somewhat sparingly soluble in cold water but dissolves readily on warming.

An analysis of this material after dialysis gave the following figures:

	%
Ash in the extract	13·95
SO_4 „ above ash	61·89
SO_4 „ extract (by hydrolysis)	21·65
SO_4 „ „ (by incineration)	8·63

The remarkably high percentage of sulphate in the ash is noticeable and also the fact that the ratio of sulphates is 2·5 : 1. It is intended to investigate this matter further.

Plumaria elegans.

This weed, of which we have not seen any previously published analyses, is characterised by an ash containing a high proportion of silica as is shown by the following:

	%	%
Ash	17·5	17·09
Silica in ash	49·76	49·61

Extracted in an autoclave at 110° the weed yields about 10 % of its weight to water. The extract evaporated to dryness over a water-bath leaves dark very brittle scales which are only partially soluble again in water even on boiling; if however the extract is evaporated in a vacuum at room temperature the resulting scales behave quite differently in water, swelling up and going into solution on standing to form a mucilaginous liquid.

Analysis of the dialysed extract of a sample obtained from Port Erin in the summer gave the following:

	%
Ash in the extract	15·94
SO_4 in the above ash	41·41
SO_4 in the extract (by hydrolysis)	9·07
SO_4 „ „ (by incineration)	6·6

Ascophyllum nodosum.

This weed was selected from among the Phaeophyceae on account of the high proportion of sulphate in its ash. The material here examined was kindly collected for us by Mr Chater near Folkestone during January. Extraction with water in an autoclave at 110° yielded a brown mucilaginous solution which was dialysed until free from adherent salts. On evaporating over a water-bath there remained opaque horny scales of a light chocolate brown colour which were insoluble in water; when however the liquid was evaporated in a vacuum at the temperature of the room the resulting solid was found to be completely soluble in cold water. The substance exhibits in a very marked degree the phenomenon of adhesion, and if dried on a glass or silica dish the scales in cracking off remove large flakes from the surface. The residual weed after exhaustive extraction with water in the autoclave still yields a quantity of substance to 5 % sodium carbonate from which it is precipitated on the addition of acid; this substance, which is presumably alginic acid, would appear to be distinct from the water-soluble

extract here described. An analysis of this substance gave the following figures:

	%
Ash in the extract	16·98
SO_4 „ above ash	55·34
SO_4 „ extract (by hydrolysis)	11·41
SO_4 „ „ (by incineration)	9·40

The presence of ethereal sulphate may be deduced from these figures in spite of the fact that the ratio of the two sulphates is only 1·2 : 1, since the known high alkalinity of the ash of this weed readily accounts for the large amount of sulphate obtained on incineration.

Corroborative evidence for the existence of an ionised salt, *i.e.* of ethereal sulphate, was furnished by placing a 1·7 % solution of the dialysed material in an osmometer when a rise of 17·5 cm. was produced in three days.

Laminaria digitata.

The following analyses were obtained from a number of samples of the weed collected at different times and places:

	I %	II %	III %	IV[1] %
Ash in the weed	32·36	48·99	13·53	13·9
SO_4 „ above ash	9·97	5·78	25·63	24·71
SO_4 „ weed (by hydrolysis)	3·12	2·11	2·69	2·15
SO_4 „ „ (by incineration)	3·22	2·83	3·46	3·43

Attention may be drawn to the striking difference in ash content between the samples, due no doubt to a difference in the amount of preliminary washing; furthermore in consequence of the large amount of saline impurities, presumably other than sulphate, in samples I and II the percentage of sulphate calculated upon the ash is in these cases comparatively low.

It is worthy of note moreover that in all cases the percentage of sulphate on hydrolysis is actually rather less than that on incineration; nevertheless the presence of an appreciable amount on hydrolysis suggests the possibility of ethereal sulphate. It seemed probable therefore that the ratio might have been thrown out by the incineration sulphate being too high, owing to the alkalinity of the ash. That this explanation is probably correct was shown by a comparison of the alkalinity of the ash of the weed with that of the ash of the extract. For this purpose samples of both weed and extract were incinerated and aqueous extracts of equal weights of the two ashes were titrated against $N/50$ hydrochloric acid with the following results:

	$N/50$ acid required
Alkali from 0·0388 g. of ash of weed	5·85 cc.
„ „ „ extract	0·24 cc.

Further confirmation was obtained by the analysis of a dialysed autoclave extract of the weed whose analysis is quoted above under column IV. This sample gave the following results:

	%
Ash in the extract	17·67
SO_4 „ above ash	50·19
SO_4 „ extract (by hydrolysis)	15·17
SO_4 „ „ (by incineration)	8·87

[1] This sample was kindly collected for us at Menai Bridge in May by Miss Purvis of University College, Bangor.

It will be seen how different a result is obtained from an analysis of the weed itself and from an extract thereof; the ratio of the two sulphates is now 1·71 : 1 owing no doubt mainly to the lower alkalinity of the ash permitting a much larger proportion of the sulphate to escape on incineration; furthermore the removal of the extraneous salts by dialysis has doubled the percentage of sulphate in the ash as compared with that of the weed. The presence of ethereal sulphate is therefore established in the water-soluble extract of *Laminaria*.

Cetraria islandica.

This lichen was selected for comparison with the above mentioned algae not because it was suspected of containing ethereal sulphate but owing to the general similarity in the physical properties of its aqueous extract with those obtained from these algae.

The following figures were obtained by analysis of a commercial sample of "Iceland Moss"; the material was air dried and not washed.

	%
Ash in the crude lichen	3·58
SO_4 " above ash	4·53
SO_4 " lichen (by hydrolysis)	0·02
SO_4 " " (by incineration)	0·16

From the above figures it is clear that this material contains no ethereal sulphate grouping and incidentally it is shown that the gelatinising properties of the substance are in no way dependent upon the existence of such a grouping.

In order to ascertain whether the mucilaginous substance extracted from Iceland moss exerted any osmotic pressure an extract was prepared and dialysed; it was then evaporated and a 1·5 % solution was placed in an osmometer but no rise whatever was recorded after three days.

Summary.

1. From the evidence furnished it appears that there exist among the marine algae a number in which calcium, and probably sodium and potassium, sulphate occur in chemical combination with an organic complex in the form of an ethereal sulphate.

2. The presence of such ethereal sulphates is established by a comparison between the amounts of sulphate contained in the ash and in the hydrolysed solution of the material concerned.

3. For the purpose of such a comparison it is best to employ a dialysed aqueous extract, although in some cases conclusive evidence is furnished by an analysis of the weed itself.

4. The ethereal sulphates above referred to have their metallic constituent freely ionised but the sulphate is masked by being in combination with a complex colloidal aggregate; they accordingly belong to the group of colloidal

electrolytes and as such have a measurable conductivity and exhibit osmotic phenomena.

5. Attention is drawn to the occurrence in the cell wall of a mucilaginous material having osmotic properties, and the question of its significance to the plant is thereby raised.

In conclusion the authors wish to express their indebtedness to the Directors of the Marine Biological Stations at Plymouth and Port Erin for kindly obtaining for them supplies of much of the material required for this investigation.

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LXXXVIII. THE INFLUENCE OF COD-LIVER OIL, LINSEED OIL AND OLIVE OIL ON THE ASSIMILATION OF CALCIUM AND PHOSPHORUS IN THE GROWING PIG.

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The literature of rickets contains many references to the use of fats and oils, especially cod-liver oil, as therapeutic agents. Gobley [1844] reported the successful use of ray's liver oil. Roloff [1866] advocated the use of oil-rich foods for the prevention of osteomalacia in farm animals. Kassowitz [1884, 1913] used cod-liver oil, and also other oils, as a vehicle for the administration of phosphorus. He did not however consider cod-liver oil of any special value *per se*, and notes that, in some cases, the administration of the oil alone had no beneficial action in curing rickets.

Schabad [1909], Birk [1909], Meyer [1913] and others have found that the administration of phosphorus in cod-liver oil increased the retention of calcium and phosphorus. On the other hand, Flieger [1897] showed that, in some cases at least, the phosphorised oil was ineffectual as a cure for rickets, and Schabad and Sorochowitsch [1911] and Schloss [1914] found that its use was not always followed by an increased retention of calcium and phosphorus.

Schloss, and Schabad and Sorochowitsch both found that an increased retention of calcium and phosphorus followed the administration of calcium salts in cod-liver oil.

The influence of fat without the addition of minerals has been studied by several workers. Kochmann and Petzsch [1911] found that the addition of fat to the diet reduced the absorption of calcium. On the other hand, Herter [1898], working with pigs, noted that, on the reduction of fat in the diet, the absorption of phosphorus from the intestine was decreased.

Orgler [1912] found that cod-liver oil increased the retention of calcium. Steinitz [1903], Rothberg [1907], Meyer [1908] and Orgler noted a decreased retention of calcium on increasing the amount of milk fat in the diet. Telfer [1921] investigated this apparent difference in the action of butter fat and cod-liver oil. In his experiments, no difference in calcium retention occurred with variations in the nature of the fat.

The recent literature on the therapeutic value of cod-liver oil in rickets need not be referred to. The work has been of a pathological, rather than a physiological nature. Interest has centred chiefly in bone lesions, and results have been judged chiefly by skiagraphic or histological examinations.

At this Institute during the past three years a series of investigations has been carried out on the mineral requirements of the growing pig, and on certain factors which affect the assimilation of the minerals. One of these investigations seemed to indicate that, in the pig at least, an important factor in the production of rickets is a lack of correspondence between the mineral content of the diet and the mineral requirements of the animal [Elliot, Crichton and Orr, 1922]. But it was found that the addition of cod-liver oil to a ration deficient in calcium led to an immediate increase in the amount of both calcium and phosphorus absorbed and retained [Orr and Husband, 1922].

In view of this finding and of the contradictory results of the previous workers referred to above, it seemed desirable to investigate further the influence of oils on the assimilation of calcium and phosphorus. It was intended to determine the relative influence of cod-liver oil, linseed oil and olive oil on the absorption and retention of these minerals. It was thought also that the results would show the influence of oil *per se*, and also whether the vitamin-rich cod-liver oil had a specific action.

Experimental Methods.

The experiments were carried out with young hog pigs two to four months old. The animals used in each experiment were taken from the same litter and were as comparable in weight as possible. They were confined in metabolism cages in which it was possible to collect urine and faeces separately. The urine was collected in 24 hour periods. The faeces were collected as soon as possible after excretion, and placed in weighed tins with tight-fitting lids. At the end of the 24 hour period the tins were again weighed, the contents thoroughly mixed, and duplicate samples taken for immediate analysis. The cages were washed out daily with distilled water, and the washings added to the urine.

To get the pigs accustomed to the metabolic cages, to the diet, and to the routine of the experiment, they were confined in the cages for about a week before the analysis of the urine and faeces began. During this period they were fed to the limit of their appetites. The amount of food fed during the experimental period was regulated according to the amount eaten during this preliminary period, and was kept constant throughout the experimental period.

Basal Diet. The basal diet used in all experiments consisted of maize meal, middlings (wheat offal), oatmeal and blood meal in the proportions stated below. Before each experiment, samples of the food-stuffs were taken from

bulk, and the percentages of N, CaO and P_2O_5 determined. The following figures are the averages for all the samples. The exact intake of N, CaO and P_2O_5 in each experiment is stated in the tables giving the results of the experiments.

Basal diet.

	Proportions	Average composition %		
		Total N	CaO	P_2O_5
Maize	10	1·48	0·008	0·633
Middlings	10	2·72	0·082	1·615
Oatmeal	10	2·15	0·077	1·084
Blood meal	1	12·96	0·226	0·380

The calcium content of this ration is very low, containing only 0·061 % of CaO, whereas the dry matter of sow's milk contains 2·37 % CaO.

Golding, Zilva, Drummond and Coward [1922] state that they had difficulty from a technical point of view to reduce the calcium intake of pigs below 0·338 % (0·47 % CaO). From their data it would appear that their sample of wheat offal contained an abnormally high percentage of calcium. The highest figure obtained in our analyses for wheat offal was 0·12 % CaO with which the figures given by Forbes [1913] and König agree.

The food was made into a thick paste with distilled water, and the animals had distilled water *ad lib.* to drink.

As it appeared from previous experiments that the influence of the oils would be found to be greatest when the mineral content of the ration was ill-balanced, and especially when there was a deficiency of calcium, it was decided not to adjust completely the mineral matter of the ration in the first series of experiments. The results of previous work however had shown that, with such a low supply of calcium, as was present in the diet, there was a danger of pathological symptoms developing during the course of the experiment. The amount of calcium was therefore increased by the addition of 50 cc. of a 20 % solution of $CaCl_2$, which is equivalent to about 2·6 g. of CaO. This raised the CaO content of the ration to between 3 and 4 g., which is not more than half of what has been found in this Institute to be the requirement of a rapidly growing young pig three or four months old.

In Exp. 3 a further adjustment of the mineral matter was made, to determine whether the effect of the oil would still be apparent with an ample supply of calcium in the food. Details of the adjustment are given with the data for the experiment. Though the excreta of each 24 hour period in Exps. 1 and 2, and of each 48 hour period in Exp. 3, were analysed separately, the averages for longer periods are given in the tables to economise space. The lengths of the periods for which the averages are given are not identical in every case. This variation is due to the fact that there was sometimes a delay of a day in introducing or withdrawing the oil if the faecal excretion of the preceding day had not been normal in amount. Even with fairly long experimental periods, it is desirable to keep in view the danger of getting a

fallacious result owing to an abnormally large or small expulsion of faeces in the 24 hours.

The following methods of analysis were used: *total nitrogen* by the Kjeldahl method; *calcium* by precipitation as oxalate and titration with $N/10$ $KMnO_4$; *phosphorus* by the Pemberton-Neumann method.

Experimental Data.

Three experiments were conducted. In each experiment one animal received cod-liver oil, one linseed oil and one olive oil.

In the first experiment the comparative effect of the three oils was tested.

This experiment was divided into three periods, the "pre-period" when the basal ration only was fed, the "oil period" when the oil was added, and the "post-period" when again only the basal ration was fed. The amount of oil added was in each case 40 cc. per day.

Experiment 1.

No. of pig	Oil	Age at beginning of experiment	Weight in kilograms: At beginning of experiment	Weight in kilograms: At end of experiment
28	Cod-liver oil	17 weeks	26·9	43·5
24	Linseed oil	17 „	26·6	44·9
26	Olive oil	17 „	24·2	38·1

Ration for Nos. 28 and 24 was 1550 g. of basal ration plus 50 cc. of 20 % $CaCl_2$ solution.

Ration for No. 26 was 1240 g. of basal ration plus 50 cc. of 20 % $CaCl_2$ solution.

It will be seen from a study of Table I that the calcium balance of all three pigs showed a progressive increase throughout the experiment.

In Exp. 2 the pre-oil period was lengthened to ascertain what course the excretion of calcium and phosphorus would follow on the basal ration alone.

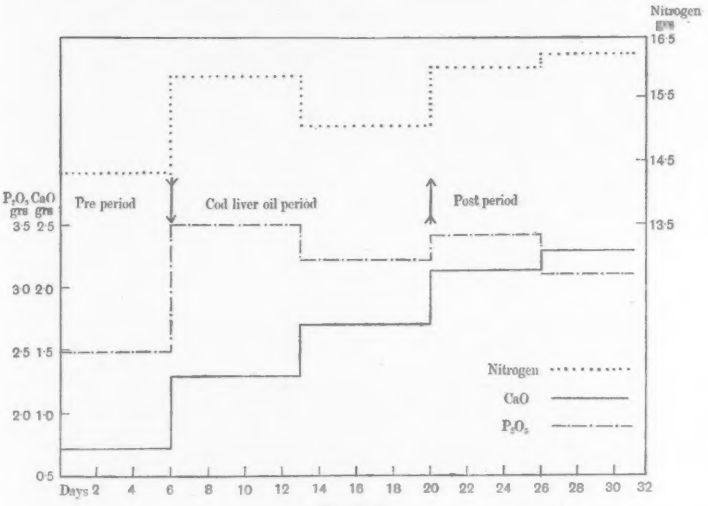
Experiment 2.

No. of pig	Oil	Age at beginning of experiment	Weight in kilograms: At beginning of experiment	Weight in kilograms: At end of experiment
67	Cod-liver oil (40 cc.)	12 weeks	20·1	28·3
68	Linseed oil (30 cc.)	12 „	19·4	28·3
70	Olive oil (30 cc.)	12 „	17·3	29·9

The ration for Nos. 68 and 70 was 930 g. of basal ration plus 50 cc. of 20 % $CaCl_2$ solution.

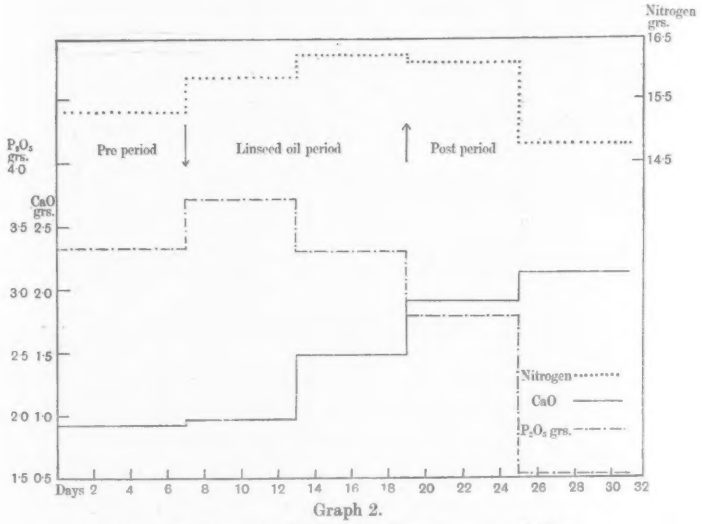
The ration for No. 67 was 1240 g. of basal ration plus 50 cc. of 20 % $CaCl_2$ solution.

In this experiment it will be noted that all three pigs showed a negative calcium balance at the end of 19 days. At this period it was decided to add



Graph 1.

Average balances for Pig No. 28. Experiment 1. Table I.



Graph 2.

Average balances for Pig No. 24. Experiment 1. Table I.

Table I. *Average daily excretions and balances in grams.*

			Average daily weight of faeces	Nitrogen					CaO					P_2O_5				
				Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
Pig No. 28. Cod-liver oil. Duration of experiment: 31 days.																		
Pre-period	6 days		919	38·70	14·54	9·85	24·39	14·31	3·54	0·32	2·50	2·82	0·72	18·07	5·30	10·29	15·59	2·48
Oil period	1st 7	„	757	„	14·56	8·29	22·85	15·85	„	0·21	2·04	2·25	1·29	„	5·58	9·00	14·58	3·49
„	2nd 7	„	891	„	13·35	10·28	23·63	15·07	„	0·14	1·70	1·84	1·70	„	5·03	9·83	14·86	3·21
Post-period	1st 6	„	803	„	13·12	9·59	22·71	15·99	„	0·12	1·30	1·42	2·12	„	4·87	9·79	14·65	3·41
„	last 5	„	792	„	13·36	9·15	22·51	16·19	„	0·12	1·14	1·26	2·28	„	4·89	10·08	14·97	3·10
Pig No. 24. Linseed oil. Duration of experiment: 31 days.																		
Pre-period	7 days		883	38·70	12·89	10·51	23·40	15·30	3·54	0·21	2·40	2·61	0·93	18·07	4·99	9·75	14·74	3·33
Oil period	1st 6	„	845	„	13·30	9·54	22·84	15·86	„	0·15	2·43	2·58	0·96	„	4·88	9·48	14·36	3·71
„	2nd 6	„	872	„	13·40	9·10	22·50	16·20	„	0·12	1·95	2·07	1·47	„	5·04	9·73	14·77	3·30
Post-period	1st 6	„	869	„	13·30	9·31	22·61	16·09	„	0·11	1·53	1·64	1·90	„	4·89	10·40	15·29	2·78
„	last 6	„	889	„	14·68	9·23	23·91	14·79	„	0·12	1·29	1·41	2·13	„	4·77	11·78	16·55	1·52
Pig No. 26. Olive oil. Duration of experiment: 28 days.																		
Pre-period	2 days		732	30·96	10·41	8·10	18·51	12·45	3·36	0·18	2·26	2·44	0·92	14·46	3·96	8·04	12·00	2·46
Oil period	1st 7	„	666	„	11·47	7·32	18·79	12·17	„	0·18	2·04	2·22	1·14	„	4·21	8·10	12·31	2·15
„	2nd 7	„	661	„	11·00	7·68	18·68	12·28	„	0·15	1·71	1·86	1·50	„	3·88	7·45	11·33	3·13
Post-period	1st 6	„	663	„	10·92	7·68	18·60	12·36	„	0·12	1·58	1·70	1·66	„	4·03	7·99	12·02	2·44
„	last 6	„	611	„	11·16	6·98	18·14	12·82	„	0·12	1·42	1·54	1·82	„	4·06	8·44	12·50	1·96

NOTE:—At the commencement of the experiment pig No. 26 received the same ration as pigs Nos. 28 and 24. Unfortunately on the first day of the collection for analyses the pig refused to consume its whole ration. The ration was therefore reduced. In the analytical data for this pig only the last two days' analyses of the pre-period were taken.

Table II. *Average daily excretions and balances in grams.*

Pig No. 70. Linseed oil. Duration of experiment: 40 days.

	Average daily weight of faeces	Nitrogen					CaO					P_2O_5				
		Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
Days 1– 5 inclusive	532	22·66	8·63	6·67	15·30	7·36	3·30	0·19	2·07	2·26	+1·04	9·45	1·51	5·18	6·69	2·76
„ 6–12 „	524	„	9·14	6·59	15·73	6·93	„	0·12	2·47	2·59	+0·71	„	1·61	5·93	7·54	1·91
„ 13–19 „	478	„	9·88	5·74	15·62	7·04	„	0·10	2·65	2·75	+0·55	„	1·35	6·22	7·57	1·88
„ 20–26 „	467	„	9·86	5·33	15·19	7·47	„	0·10	3·26	3·36	−0·06	„	1·09	6·82	7·91	1·54
„ 27–33 ⟶	481	„	9·82	5·05	14·87	7·79	„	0·12	3·27	3·39	−0·09	„	1·00	7·05	8·05	1·40
„ 34–40	467	22·89	9·21	4·59	13·80	9·09	3·13	0·11	3·06	3·17	−0·04	9·44	1·09	7·06	8·15	1·29

Pig No. 67. Cod-liver oil. Duration of experiment: 40 days.

Days 1– 7 inclusive	638	30·21	10·35	8·52	18·87	11·34	3·48	0·12	2·72	2·84	+0·64	12·59	2·42	6·86	9·28	3·31
„ 8–13 „	621	„	11·00	7·47	18·47	11·74	„	0·13	3·18	3·31	+0·17	„	1·99	7·59	9·58	3·01
„ 14–19 „	716	„	11·48	7·89	19·37	10·84	„	0·15	3·69	3·84	−0·36	„	1·93	9·06	10·99	1·60
„ 20–22 ⟶	726	„	11·81	8·03	19·84	10·37	„	0·19	3·77	3·96	−0·48	„	1·73	9·36	11·09	1·50
„ 23–28 „	446	20·14	9·49	5·34	14·83	5·31	2·32	0·12	2·03	2·15	+0·17	8·40	2·45	5·39	7·84	0·56
„ 29–34 „	347	„	8·43	4·72	13·15	6·99	„	0·07	0·71	0·78	+1·54	„	2·29	3·58	5·87	2·53
„ 35–40 „	237	15·25	7·35	3·25	10·60	4·65	1·70	0·05	0·49	0·54	+1·16	6·30	2·12	2·63	4·75	1·55

Pig No. 68. Olive oil. Duration of experiment: 38 days.

Days 1– 4 inclusive	470	22·66	9·07	6·24	15·31	7·35	3·30	0·14	2·11	2·25	+1·05	9·45	1·89	5·15	7·04	2·41
„ 5–10 „	429	„	9·30	5·55	14·85	7·81	„	0·12	2·17	2·29	+1·01	„	2·08	5·03	7·11	2·34
„ 11–16 „	474	„	9·77	5·74	15·51	7·15	„	0·12	3·10	3·22	+0·08	„	1·84	6·20	8·04	1·41
„ 17–20 ⟶	432	„	9·80	5·29	15·09	7·57	„	0·13	3·28	3·41	−0·11	„	1·50	6·41	7·91	1·54
„ 21–26 „	454	„	10·14	5·74	15·88	6·78	„	0·15	3·29	3·44	−0·14	„	1·91	6·54	8·45	1·00
„ 27–32 „	401	„	10·17	5·12	15·29	7·37	„	0·15	2·36	2·51	+0·79	„	2·17	5·33	7·50	1·95
„ 33–38 „	437	22·98	10·15	5·33	15·48	7·50	3·07	0·14	2·24	2·38	+0·69	9·43	2·19	5·47	7·66	1·77

NOTE. ⟶ indicates the introduction of the oil.

Table III. *Average daily excretions and balances in grams.*

Pig No. 669. Cod-liver oil. Duration of experiment: 20 days.

	Average daily weight of faeces	Nitrogen					CaO					P_2O_5				
		Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
Days 1– 6 inclusive	490	22·77	9·56	5·91	15·47	7·30	3·54	0·11	2·56	2·67	+0·87	9·77	1·77	6·17	7·94	1·83
,, 7–10 ,,	467	,,	8·87	5·78	14·65	8·12	,,	0·15	2·91	3·06	+0·48	,,	1·84	6·19	8·03	1·74
,, 11–14 ——→	459	22·88	10·75	5·55	16·30	6·58	3·51	0·12	3·74	3·86	−0·35	9·75	2·34	7·02	9·36	0·39
,, 15–18 ,,	517	22·98	10·12	6·07	16·19	6·79	3·49	0·08	2·50	2·58	+0·91	9·73	2·30	5·87	8·17	1·56
,, 19–20 ,,	643	,,	11·02	6·50	17·52	5·46	,,	0·11	1·79	1·90	+1·59	9·73	2·20	5·33	7·53	2·20

Pig No. 670. Olive oil. Duration of experiment: 40 days.

Days 1– 6 inclusive	515	22·77	10·25	6·52	16·77	6·00	14·59	0·64	11·92	12·56	+2·03	9·77	0·37	7·57	7·94	1·83
,, 7–12* ,,	512	22·98	10·93	6·33	17·26	5·72	14·55	0·61	13·03	13·61	+0·91	9·72	0·38	8·38	8·76	0·96
,, 13–18 ,,	510	,,	11·19	5·96	17·15	5·83	14·52	0·50	13·27	13·77	+0·75	13·29	0·59†	11·25†	11·84†	1·45†
,, 19–22 ——→	557	,,	9·74	5·83	15·57	7·41	,,	0·36	14·17	14·53	−0·01	,,	0·40	11·91	12·31	0·98
,, 23–28 ,,	540	,,	11·03	5·42	16·45	6·53	,,	0·45	13·68	14·13	+0·39	,,	0·62	11·34	11·96	1·33
,, 29–34 ,,	503	,,	10·63	5·21	15·84	7·14	,,	0·39	12·79	13·18	+1·34	,,	0·56	10·69	11·25	2·04
,, 35–40 ,,	471	,,	10·13	5·11	15·24	7·74	,,	0·44	12·68	13·12	+1·40	,,	0·66	10·16	10·82	2·47

Pig No. 671. Linseed oil. Duration of experiment: 42 days.

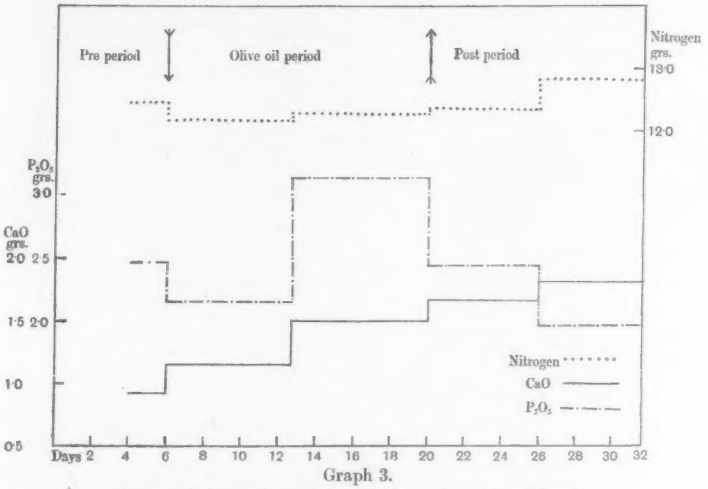
Days 1– 4 inclusive	480	22·78	11·84	6·30	18·14	4·64	3·54	0·12	3·17	3·29	+0·25	9·77	2·72	6·47	9·19	0·58
,, 5–10 ,,	458	,,	10·82	5·47	16·29	6·49	,,	0·13	3·07	3·20	+0·34	,,	2·52	6·21	8·73	1·04
,, 11–16 ,,	445	22·84	10·88	5·23	16·11	6·73	3·52	0·16	3·60	3·76	−0·24	9·76	2·44	6·76	9·20	0·56
,, 17–20 ——→	428	22·98	10·80	5·08	15·88	7·10	3·49	0·17	3·82	3·99	−0·50	9·73	2·05	7·03	9·08	0·65
,, 21–26 ,,	446	,,	10·26	5·10	15·36	7·62	,,	0·15	3·88	4·03	−0·54	,,	1·83	7·17	9·00	0·73
,, 27–32 ,,	480	,,	10·26	5·36	15·62	7·36	,,	0·14	3·74	3·88	−0·39	,,	1·98	7·39	9·37	0·36
,, 33–38 ,,	474	,,	10·68	5·01	15·69	7·29	,,	0·16	3·63	3·79	−0·30	,,	2·31	7·00	9·31	0·42
,, 39–42 ,,	454	,,	10·63	4·79	15·42	7·56	,,	0·15	3·54	3·69	−0·20	,,	2·30	6·88	9·18	0·55

——→ indicates the introduction of the oil.

* $Ca_3(PO_4)_2$ introduced on this day.

† Average for four days only, figures for the first two days after the addition of $Ca_3(PO_4)_2$ being rejected.

40 cc. of cod-liver oil to the ration of pig No. 67 and 30 cc. of olive oil to the ration of pig No. 68. Pig. No. 70 was continued on the basal ration alone for a further period of 14 days as a control, to ascertain whether the negative calcium balance would continue.



Graph 3.

Average balances for Pig No. 26. Experiment 1. Table I.

At the end of this time 30 cc. of linseed oil were added to the ration of this pig. It will be seen from Table II that the addition of the oil to the ration resulted in a rapid conversion of the negative calcium balances to positive balances in the case of pigs Nos. 67 and 68, and in a lowering of the negative balance of pig No. 70.

Experiment 3.

No. of pig	Oil	Age at beginning of experiment	Weight in kilograms: At beginning of experiment	Weight in kilograms: At end of experiment
669	Cod-liver oil (30 cc.)	14 weeks	19·7	25·8
670	Olive oil (30 cc.	14 ,,	20·6	31·0
671	Linseed oil (30 cc.)	14 ,,	25·1	35·5

Ration for Nos. 669 and 671 was 930 g. of basal ration plus 50 cc. of 20 % $CaCl_2$ solution.

Ration for No. 670 was 930 g. of basal ration plus 26 g. of chalk and 5 g. of NaCl.

In Exps. 1 and 2 the mineral matter of the diet was ill-balanced, especially with regard to calcium, sodium and chlorine, which were markedly deficient. In this experiment those deficiencies were made good in the case of one of the pigs, No. 670, by the addition to the day's ration of 26 g. of $CaCO_3$, and 5 g. of NaCl. On the 12th day of the experiment 8 g. of tricalcium phosphate

were substituted for 6 g. of the $CaCO_3$, to adjust the phosphorus content of the ration. This ration thus adjusted contained the mineral ingredients in amounts approximately equal to those contained in an amount of sow's milk of equal caloric value. Of course, though these various minerals were present roughly in the same proportion as those found in sow's milk, it must not be assumed that they were present in the same utilisable form.

Discussion of Results.

It will be seen from Tables II and III that prior to the addition of oil there was a progressively increasing excretion of both calcium and phosphorus. This occurred in every pig used in these two experiments. In every case where the pre-period was prolonged, the calcium balance became negative, and the phosphorus balance correspondingly reduced.

The increased excretion is in the faeces. The urinary excretion of both calcium and phosphorus in pig No. 70 decreased in the pre-period. This happened also in pigs 68 and 670.

All the pigs were in the metabolism cages for a period of not less than seven days before analysis of the excreta was begun. It is most probable that during this preliminary period also, there was a decrease in the assimilation and retention of both calcium and phosphorus. The amounts retained even at the beginning of analysis were small compared with the normal requirement for growing pigs, which has been found here to be about 4–6 g. per day for pigs of the age used in the experiments.

Correlated with the decreased retention of calcium and phosphorus, a loss of appetite was noted. As the experiments proceeded, it became difficult to get the pigs to consume the daily ration. Pig No. 67 (Exp. 2), shortly after the introduction of cod-liver oil into the ration, could not be induced to consume the full ration, and similarly pig No. 669 (Exp. 3) finally refused its food, and work with it had to be abandoned.

A notable exception to this loss of appetite was pig No. 670, which had the adjusted mineral matter. Although the calcium balances showed the same downward course as with the other pigs, it was always eager for its food. Shortly after the experiment began its appetite improved and it would have eaten much more than the ration which satisfied it in the preliminary period.

It is interesting to note that this was the only pig used in these two experiments, which at the end of the experiment was in good condition. It was still active and apparently in good health. All the other five were beginning to show signs of ill health.

There is not sufficient data available for a profitable discussion of the cause of this decreased assimilation and retention of calcium and phosphorus.

The point of importance in the present investigation is that the diet on which the oils were superimposed was a diet on which normal assimilation and retention of calcium and phosphorus did not occur. The close confinement

in the metabolism cages was very probably a contributing factor, but the fact that, under the same conditions and with the same animals, assimilation and retention markedly increased on the introduction of oil to the diet demonstrates quite clearly the beneficial effect of oil on the metabolism of these minerals.

Oil period and post-oil period. The introduction of the oil, whether cod-liver, linseed or olive oil, is followed in all cases by a definite increase in the retention of calcium and phosphorus. In Exp. 1 where the pre-period was too short for the animals to reach a negative calcium balance, the calcium retention within seven days exceeded that of the pre-period. In general, the effect on calcium is more marked than on phosphorus. There is a correlation between calcium and phosphorus retention in growing animals, as has been noted by Fraps [1918] and others. This has been observed in all the metabolic experiments with growing pigs done in this Institute.

It has been noted above that, in the pre-period, the increased excretion of calcium and phosphorus that occurred when the pigs were continued on the basal ration, was due to increased elimination in the faeces. The increased retention on administration of the oil is brought about by decreased elimination in the faeces. Compared with the faecal excretion, the effects of variations in the urinary excretion on the calcium and phosphorus balances are unimportant.

Unfortunately it is impossible to differentiate the faecal excretion into the unabsorbed portion and the excreted portion; and consequently there is no direct evidence to settle the question as to whether the oils merely cause an increased absorption of these minerals, or cause an increased retention after they are absorbed.

It has been generally believed that calcium salts are excreted chiefly by the large intestine. The fact that calcium salts, when injected intravenously, appear in the intestine, seems to support this view (a review of this work is given by Von Noorden [1907]).

The results of the recent interesting studies of Telfer [1922], however, make it appear doubtful whether, normally, calcium or phosphorus is excreted into the intestine. He regards the mineral matter of the faeces in his experiments as chiefly, if not entirely, unabsorbed residues.

If the mineral matter in the large intestine is to be regarded as unabsorbed residues, then the effect of oil is exercised in the alimentary tract. The increased absorption must however be due to something more than a mere physical or chemical change in the contents of the intestine, because in Exp. 1, where the post-period was continued for 12 days, increased retention of calcium was continued after the elimination of the oil from the diet. Indeed, retention of calcium was greater in the days 7 to 12 of the post-period than during the administration of the oil.

Comparative effect of the three oils.

A comparison of the effects of the three different oils is of interest. In Exp. 1 when the pre-period was short, and the three animals were still

retaining a considerable amount of calcium and phosphorus, the oils appear about equally potent in increasing the retention of both minerals.

In Exps. 2 and 3, when the animals had got into a pathological condition, as shown by the negative calcium balances, cod-liver oil was the most potent in increasing the assimilation and retention of the minerals. Of the other two, olive oil appears from a comparison of pigs Nos. 68 and 670 with pig. No. 671, to be greater in its action than linseed oil. On the other hand, there was a marked improvement in the appetite of the animals when given linseed oil or olive oil, but the introduction of cod-liver oil resulted in a depression of the appetite. Hart, Steenbock and Hoppert [1922] noted a similar effect of cod-liver oil on the appetite of goats.

The fact that olive oil and linseed oil, which contain little or no fat-soluble A, are almost as effective in stimulating the assimilation and retention of calcium and phosphorus as the vitamin-rich cod-liver oil, indicates that the action on the metabolism of the minerals is not due, entirely at all events, to the vitamin A.

This conclusion is supported by the work of McCollum, Simmonds, Becker and Shipley [1922] who tested a number of fats for their power to stimulate healing in rickets. They found that cod-liver oil oxidised for 12 hours to destroy fat-soluble A, still retained its power to cause the deposition of calcium in the bones of young rats suffering from rickets. A similar result was got with coconut oil, which is also deficient in vitamin A.

It is most probable that oil *per se* has an influence on the absorption of minerals from the intestine, though the above workers found that olive oil fed to rats suffering from rickets did not raise the efficiency of the tissues in utilising calcium. In the light of our results it is probable that a positive result would have been obtained by these workers if the oil had been introduced earlier, or if the experiment had continued over a longer period. Unfortunately in these experiments with rats there are no data to show the actual mineral balances.

In our experiments, when the animals had been showing a negative calcium balance for some time, the response when receiving linseed oil was not so rapid as in the case of animals still assimilating and retaining these minerals (cf. pig No. 671, Exp. 3 with pig No. 24 of Exp. 1).

The results obtained here make it clear that the effect produced on a growing animal by the introduction of cod-liver oil cannot be ascribed to the vitamin present in the oil.

Note. It will be seen from the tables and graphs that the nitrogen metabolism of each pig was also followed. These results seem to indicate some difference in the action of the three oils on the nitrogen metabolism. This point is being further investigated, and it is hoped to discuss the combined results in a future communication.

Conclusions.

1. Pigs confined in metabolism cages and fed on a grain ration show a progressively decreasing capability of absorbing or retaining calcium and phosphorus.

2. The introduction of cod-liver oil, linseed oil or olive oil into the grain ration leads to increased assimilation and retention of both minerals.

3. When the animal is still retaining appreciable amounts of calcium and phosphorus, the three oils are about equally potent in their action.

4. When the animal shows a negative calcium balance, both cod-liver oil and olive oil rapidly convert the negative calcium balances to positive balances. Linseed oil is much slower in its action.

5. When the animal shows a negative calcium balance the introduction of cod-liver oil into the ration causes digestive disturbances.

6. The beneficial effect of an addition of oil to a grain ration on the calcium and phosphorus metabolism appears to be largely due to the oil *per se* irrespective of its content of vitamin A.

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LXXXIX. CARBOHYDRATE AND FAT METABOLISM IN YEAST.

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PART I. THE FORMATION OF FAT FROM NON-NITROGENOUS SUBSTANCES OTHER THAN SUGARS.

Forty-five years ago Nägeli [1878, 1879] examined the production of fat by moulds and yeasts and found that the fat content of these organisms could be increased from 5 % to 12 % when they were grown in a well-aerated medium rich in carbohydrate and poor in nitrogen. During the war Lindner [1922] and his co-workers applied similar conditions of growth to the organism *Endomyces vernalis*, and the fat content of this was so successfully raised that it was used in Germany to provide rations both of fat and protein. Lindner also made a histological investigation of yeast cells grown under similar conditions of aeration in a medium rich in carbohydrate and poor in nitrogen. He describes as the first stage of fat formation a granular appearance of the plasma, the granules coalescing gradually to form fat globules, which could be stained with appropriate reagents, *e.g.* osmic acid, Sudan III or alkanna. Solutions of sugar and of alcohol were found to be suitable media from which the yeast cell could increase its quantity of stored fat. Superficial cultures of yeast cells were also found to become laden with fat globules when submitted to the action of alcohol vapour. Berzfeld [1922] described the power of the yeast cells to stain with alkanna and Sudan III as a criterion of age, quality and degeneration and found it was not till the third or fourth generation that the fat in the yeast cell could be shown by staining. There are also a number of patents dealing with the changes produced by different media on the fat content of yeast. Quantitative data have been however almost entirely lacking and even the relative efficiency of sugar and alcohol as sources from which the yeast cell could produce fat does not seem to have been determined.

Nägeli was aware that the direct extraction of dried yeast by ether did not remove all the fat it contained, but subsequent workers do not appear to have recognised this. In an earlier communication by one of us it was shown that the amount of fat which could be obtained from a sample of yeast depended on the method of extraction used [Smedley MacLean, 1922]. The proportion of the total fat present which could be extracted directly from dried yeast in a Soxhlet apparatus by ether also varied according to the previous history of the yeast. From yeast previously incubated in a well-oxygenated solution of sugar only a small proportion of the fat it contained was separated by direct extraction, but if the yeast was first hydrolysed by boiling it for two

hours with normal acid and the residue dried and extracted with ether a much larger quantity of fat was obtained than by the direct extraction process; the acid value of the fat was only slightly raised, showing that hydrolysis had only taken place to a very small extent. If the fat was estimated in an old sample of yeast both by ether extraction of the dried yeast and by extraction of the dried residue after hydrolysis there was much less difference between the results obtained by the two methods than in the case of the recently incubated yeast described above. The fat content of all samples of yeast was however found to be higher when determined after hydrolysis of the yeast.

The hydrolysis method has also the advantage of allowing the carbohydrate content of the yeast to be determined at the same time in the filtrate so that the relation between the fat and carbohydrate contents of the yeast may be conveniently studied. By the method of acid hydrolysis any lipin present in the fat would be partly decomposed and glycerophosphoric acid separated so that if any lipin were present the fat obtained by direct extraction would lose a fraction of its weight during the hydrolysis.

It is clear that care must always be taken to differentiate between a real and an apparent increase of fat in the cell. Since the fat when first formed in the yeast cell appears to be in a condition in which it cannot be directly extracted by ether, the effect of incubating the yeast in a certain medium might conceivably be to produce an apparent increase of fat by altering the state in which the fat is held in the cell and rendering it capable of being extracted by solvents. If the yeast be first heated with dilute acid as described this difficulty is eliminated.

It seemed to us that it would be of interest to obtain quantitative data as to the amount of fat stored in yeast when the yeast is incubated in solutions of various non-nitrogenous organic substances and we investigated therefore the action in this respect of the following:

(1) simple organic substances such as alcohols and the sodium salts of fatty acids;

(2) the sugars: lactose, glucose, fructose, sucrose and maltose;

(3) sugar solutions with the addition of alkali phosphates.

Method of experiment.

Samples of the yeast were weighed out for the determination of moisture and nitrogen and four quantities each of 12·5 g. were also weighed; two of these samples were hydrolysed by boiling each with 100 cc. of *N* HCl for two hours under a reflux condenser; the mixture was filtered and the residue well washed with hot water; to the filtrate and washings 80 cc. of *N* NaOH were added and about 30 cc. of "dialysed iron" and the mixture was made up to 1 litre. After filtering, the reducing power of the filtrate was determined by Bertrand's method and the total reducing carbohydrate present after hydro-

lysis calculated as glucose for the purpose of comparison. The residue of yeast cells after boiling with the acid was air dried over-night on the filter and then extracted with ether for 48 hours in a Soxhlet apparatus. After evaporation of the ether the residue was taken up with dry ether, filtered into a weighed flask, the ether evaporated and finally the ether-soluble substance dried to constant weight in a vacuum desiccator. The two remaining quantities of 12·5 g. of yeast were added each to a litre of the solution to be tested contained in a Winchester quart bottle. Into one of these a tube was fitted through the cotton-wool plug and after placing in the incubator at 25–26°, a current of oxygen was passed through for 45 hours; the other was placed in the incubator, no oxygen being passed through. At the end of the experiment the contents of each were centrifuged and the yeast filtered on a Buchner funnel, well washed and weighed. Samples were taken for the determination of moisture and nitrogen and the remainder hydrolysed and treated exactly as described for the original yeast.

The substance soluble in the dry ether was regarded as fat; the acid value of the fat obtained in this way was found to be slightly higher than that of the fat directly extracted by ether from the dried yeast. It is possible that the slight increase is due to acid liberated from the lipins present. The yeast sterol, previously identified as ergosterol [Smedley MacLean and Thomas, 1920], is always present with the fat. In our earlier experiments the pressed yeast used was supplied by a yeast dealer, but the results obtained were not consistent; the age of the yeast seemed to be an important factor since the power of a specimen of pressed yeast to form fat fell off rapidly when the yeast was kept in the cold room for one or two days before being used. We subsequently worked with an ale yeast from 84 to 110 hours old supplied directly from a brewery where we could rely on the yeast being grown under approximately constant conditions; this yeast we found contained appreciably less sterol than that used in our earlier experiments.

The effect on the fat content of yeast produced by incubating it in solutions of simple organic substances.

The effect of incubating yeast for 45 *hours in water at* 25° *with and without oxygenation* was first ascertained as a basis of comparison for future experiments. We found that 12·5 g. of brewery yeast incubated for 45 hours at 25° in a litre of water and oxygenated throughout this period lost about two-thirds of the carbohydrate originally present and about a seventh of the protein; the quantity of fat however increased by from 50 to 100 % of that originally present. If the oxygen was not passed there was slightly less loss of carbohydrate and of protein and no increase of fat. These experiments confirm the results previously arrived at [Smedley MacLean, 1922] that a yeast with a high carbohydrate content loses carbohydrate and gains fat when incubated in oxygenated water. The specimens of pressed yeast ex-

amined at an earlier date had been kept for some time and did not show the increase of fat after incubation in water, but as stated above we found that the fat-forming power of a yeast rapidly fell off on keeping the yeast in the cold room.

In subsequent experiments carried out with 12·5 g. of yeast containing approximately 0·1 g. of fat we have regarded increases of fat up to 0·1 g. as capable of being formed from materials originally present in the cell since this increase could be obtained by incubating the yeast in oxygenated water. If the amount of fat was increased beyond this we have attributed it to the influence of the substance dissolved in the medium.

The substances investigated were the alcohols, methyl, ethyl, propyl, *iso*-propyl, butyl and *iso*-amyl, glycol and glycerol; acetone, acetaldehyde and the sodium salts of the following acids, formic, acetic, butyric, lactic and pyruvic. The method of procedure was to make up a solution of the substance to be tested, generally of decinormal strength and to incubate the yeast in this as described above.

We found we could divide these substances into three classes:

(*a*) *Those producing a definitely inhibitory effect*, no increase of fat being observed or an amount so small as to be within the region of experimental error. Under this heading we include methyl, propyl, butyl and *iso*-amyl alcohols, that is to say that all the normal fatty alcohols tested, with the exception of ethyl alcohol, appear to be definitely injurious to the formation of fat when supplied in decinormal solution, less fat being found than when the yeast was incubated under similar conditions in water. We found also that acetaldehyde supplied in 0·6 % concentration produced little change in the fat and carbohydrate content; it appeared to be injurious to the yeast and there was a marked increase in the loss of protein. These results contrast with those obtained by Haehn [1923] in his recently published paper on *Endomyces vernalis*, where a marked increase of fat was observed when the organism was supplied with acetaldehyde in a concentration of 1 %, alkali phosphates being also present in the solution.

(*b*) *Substances, solutions of which behave similarly to water* and which may therefore be assumed to have no influence on the production of fat. Decinormal solutions of the sodium salts of formic, propionic and butyric acids, of *iso*-propyl alcohol and acetone, glycol in 0·5 % and glycerol in both 0·5 and 5 % solutions belong to this class. Small increases of fat were recorded quite comparable with those observed when the yeast was incubated in water and the amount of carbohydrate found at the end of the period of incubation was also of the same order in both cases.

(*c*) *Substances which produce an increase of fat greater than that observed after incubation of yeast in water.* To this class belong ethyl alcohol, and the sodium salts of acetic, pyruvic and lactic acids.

Ethyl alcohol. Our experiments showed that if yeast were incubated for 45 hours in a 0·5 or 0·6 % solution of ethyl alcohol, the increase of fat in the

yeast at the end of the incubation period was more than after incubation in water for the same time; in one experiment only did we get an increase of fat comparable with that obtained after yeast had been incubated in a sugar solution, but in six other experiments carried out under as far as we knew similar conditions the increase was very much smaller. If we increased the percentage of alcohol in the medium, the amount of fat stored showed no corresponding increase but a diminution.

Lindner and Unger [1919] have described the effect of alcohol vapour on different brewery yeasts and have shown that it brings about the deposition of globules of oil in the cell. It is possible that this result may be partly due to a change in the state of fat in the cell whereby the separation of the fat already present in the cell is affected. Also, that the microscopic changes which Lindner himself has described, the preliminary granulation of the cell plasma gradually followed by the collection of the granules in globules, may be hastened by the effect of the alcohol vapour. Lindner [1922] found that both with brewery yeasts and with *Endomyces vernalis* the assimilation of alcohol leads to the production of fat. In our experiments with brewery yeast alcohol is very much less effective as an aid to the formation of fat than any of the fermentable sugars. In 0·5 % solution, ethyl alcohol is as effective as a 0·5 % sugar solution in producing fat, but the amount of fat formed does not increase when the concentration of alcohol in the solution is increased and alcohol cannot therefore be put into the same category as the sugars when classified according to its fat-forming power.

Sodium acetate we found rather more effective as a fat former than ethyl alcohol, but again when the concentration of the acetate was increased above decinormal there was a diminished increase of fat. The same held also for sodium lactate and for sodium pyruvate.

These four substances can certainly be classed together as having a distinct though small influence on the production of fat. We do not however feel justified in saying that from these experiments there is any satisfactory evidence that any one of these substances can be regarded as an intermediate stage in the process of fat formation.

The increase of fat observed after incubating yeast in oxygenated water clearly proves that fat is formed from the carbohydrate held in the cell, since the loss of protein is insufficient to account for it. It may be that by the assimilation of such substances as ethyl alcohol or sodium acetate the carbohydrate storage is directly influenced and the fat formation is only indirectly influenced, and it is interesting to note that in these cases the carbohydrate content of the yeast is higher after incubation in the oxygenated than in the non-aerated solution and the amount stored may even exceed that present in the original sample of yeast. Except for one experiment with alcohol in which an abnormally high fat content was obtained and which we have never been able to repeat, the largest increase of fat was obtained after incubation in oxygenated sodium acetate solution where we got an increase of from 0·1

Table I.

Medium	Original yeast: Age of yeast. Hours	Original yeast: Carbohydrate g.	Original yeast: Fat g.	Original yeast: Protein g.	Oxygenated yeast: Carbohydrate g.	Oxygenated yeast: Fat g.	Oxygenated yeast: Protein g.	Non-oxygenated yeast: Carbohydrate g.	Non-oxygenated yeast: Fat g.	Non-oxygenated yeast: Protein g.
Water	88	1·21	0·0897	1·31	0·39	0·1420	1·12	0·50	0·0912	1·11
	110	0·75	0·1042	1·20	0·30	0·1590	1·02	0·31	0·0932	1·08
	70	1·52	0·1039	1·15	0·57	0·1978	0·98	0·65	0·1051	1·05
Substances, incubation in solutions of which produces less increase of fat than incubation in water at 25°.										
Alcohols:										
Methyl *M*/10	106	0·64	0·1229	1·29	0·27	0·1463	1·26	0·33	0·1200	—
Propyl *M*/10	83	0·72	0·1027	1·25	0·30	0·1159	1·08	0·37	0·1049	1·14
Butyl *M*/10	102	0·70	0·0817	1·40	0·32	0·0898	1·27	0·31	0·0784	1·18
iso-Amyl *M*/10	108	0·65	0·1107	1·37	0·28	0·1096	1·18	0·31	0·1127	1·18
Acetaldehyde:										
0·1 %	108	0·91	0·1050	1·46	0·37	0·1500	1·33	0·53	0·1110	1·41
0·6	78	0·99	0·0978	1·26	0·92	0·0890	0·82	0·87	0·0906	0·75
Substances, solutions of which act like water.										
Na formate *M*/10	86	1·52	0·0918	1·29	0·56	0·1841	1·17	0·46	0·0935	1·30
Na propionate *M*/10	62	1·20	0·1176	1·22	0·32	0·1856	1·10	0·36	0·1150	1·09
Na butyrate *M*/10	—	0·47	0·0981	1·29	0·27	0·1849	1·15	0·37	0·1187	1·17
	84	1·39	0·0920	1·30	0·70	0·1625	1·20	0·74	0·1033	1·24
iso-Propylalcohol *M*/10	106	0·98	0·0893	1·32	0·38	0·1745	1·01	0·37	0·0968	1·20
Acetone *M*/10	85	1·22	0·1006	1·43	0·57	0·1667	1·26	0·65	0·1040	1·33
Glycol 0·5 %	85	1·29	0·0920	1·35	0·39	0·1497	1·21	0·41	0·0816	1·20
„ 0·5	101	0·74	0·1092	1·44	0·36	0·1404	1·29	0·42	0·1010	1·34
Glycerol 0·5 %	84	1·15	0·1228	1·20	0·46	0·2086	1·10	0·47	0·1073	1·18
„ 5·0	72	1·19	0·1167	1·23	0·43	0·1997	1·11	0·33	0·1148	1·13
Substances, solutions of which produce marked increase of fat.										
Ethyl alcohol:										
0·5 %	103	1·27	0·0862	1·08	0·53	0·2610	1·24	0·46	0·1032	1·22
0·5	83	0·83	0·1065	1·36	0·50	0·2417	1·26	0·44	0·1144	1·22
0·5	86	0·89	0·1065	1·24	0·71	0·5221	1·16	0·54	0·0984	1·20
0·5	83	1·12	0·1163	1·23	0·66	0·1889	1·19	0·74	0·1254	1·20
0·64	130	1·03	0·1110	1·34	0·43	0·2636	1·22	0·38	0·1125	1·22
2·0	106	0·97	0·1122	1·34	0·41	0·2225	1·24	0·33	0·1064	1·21
4·0	76	1·30	0·1140	1·24	0·47	0·1952	1·09	0·47	0·1032	1·15
Sodium acetate:										
0·6 %	—	0·51	0·1816	1·48	1·01	0·3930	1·35	0·40	0·1882	1·45
	96	0·60	0·1084	1·33	0·34	0·3475	1·16	0·22	0·1062	1·19
	84	1·21	0·0846	1·32	1·10	0·2136	1·22	0·50	0·0897	1·21
1·0	83	1·12	0·1034	1·23	0·41	0·2182	1·11	0·43	0·1059	1·35
2·0	106	1·14	0·0927	1·36	0·34	0·2172	1·25	0·42	0·0945	1·26
5·0	105	0·80	0·1126	1·27	0·28	0·1417	0·93	0·26	0·1049	0·86
Sodium lactate:										
0·90 %	82	1·10	0·1208	1·31	0·42	0·2470	1·23	0·30	0·1180	1·09
	107	0·80	0·1058	1·31	0·39	0·1664	1·15	0·37	0·1080	1·08
2·0	84	1·31	0·1035	1·23	0·51	0·2113	1·16	0·43	0·1070	1·20
Sodium pyruvate:										
0·88 %	82	1·31	0·0945	1·29	0·53	0·2291	1·19	—	—	—
	94	0·89	0·1026	1·39	0·47	0·2204	1·20	—	—	—
	96	1·14	0·1006	1·33	0·60	0·2461	1·32	0·69	0·1185	1·33
	81	1·05	0·0932	1·31	0·40	0·2270	1·24	0·38	0·1153	1·17

to 0·35 g., an increase of about 10 % on the dry weight of the yeast. Since in some of the acetate experiments there was at the end of the experiment an actual increase in the amount of the carbohydrate stored in the cell, it

follows that the cell was able to build up carbohydrate from the acetate supplied and some of the newly formed carbohydrate may have been converted to fat.

It may be that we cannot present any simple intermediate substance to the cell under suitable conditions for the cell to make use of it; it does not necessarily follow that these substances may not occur in the cell as intermediate substances. It is interesting to note that Stephenson [1922] found that sodium acetate exerted a beneficial effect on fat formation in the Timothy Grass Bacillus.

PART II. THE RELATIVE EFFICIENCY OF THE DIFFERENT SUGARS IN PRODUCING STORAGE OF CARBOHYDRATE AND OF FAT.

When yeast is incubated in an oxygenated solution of fermentable sugar, the fat content is increased.

In stating our results we have recorded the actual amounts of fat, carbohydrate and protein in the yeast before incubation and compared these with the amounts present when the same weight of the yeast has been incubated in a solution of the substance under investigation. A comparison of the percentages of fat and carbohydrate found in the samples of yeast before and after incubation calculated on the dry weight of the yeast may give rise to misleading conclusions. No nitrogen being present in the nutrient solutions, an increase in weight must be due to an increase in the amount of fat or carbohydrate present. If the latter be increased, although no fat may have been lost, the percentage of fat will be diminished and similarly a diminution of the carbohydrate content produces an apparent increase of fat when expressed as fat percentage although the total amount of fat present may be less than at the beginning of the experiment.

The method of carrying out the experiments was the same as that described above for the simpler carbon compounds (cp. p. 721). The pressed yeast with which we worked showed comparatively small variations in the percentage of fat it contained. The 12·5 g. used for each experiment contained approximately 0·1 g. fat, the amount in the vast majority of samples lying between 0·08 and 0·1 g. and the results of the experiments were practically unaffected by these small differences.

The variations in the carbohydrate contents of the original pressed yeast were very much wider and seem to influence to some extent the final figures for the carbohydrate when the yeast is incubated in a 0·5 or 1·0 % solution of sugar. In stronger solutions the influence does not seem to be perceptible. A survey of the accompanying tables will however show that the amounts of carbohydrate contained in a fixed weight of yeast after it has been incubated in a sugar solution are remarkably constant for any given concentration of solution and must depend mainly on this factor.

With regard to the nature of the fat formed it appears to be closely similar to that present in the original yeast; it shows the same power of remaining

concealed in the cell until after the yeast has been hydrolysed with dilute acid, which has already been described. The saponification number of the fat from different samples lay between 139 and 186 and the Hübl Iodine Values varied considerably, the limits being from approximately 80 to 132. These differences may be partly explained by variations in the amount of ergosterol present and partly perhaps by oxidation, as the newly formed fat

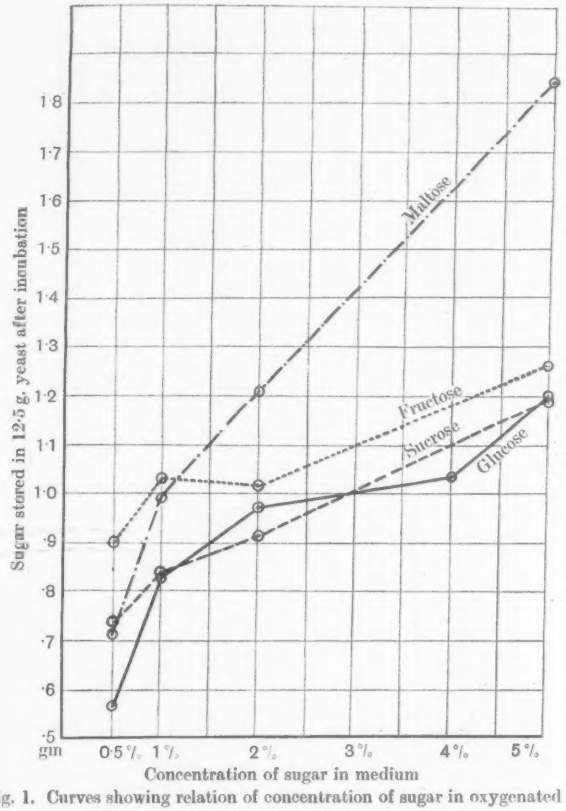

Fig. 1. Curves showing relation of concentration of sugar in oxygenated medium to amount of carbohydrate stored by yeast.

seemed very susceptible to oxidation. We were unable to detect any consistent differences between the fat formed under different conditions and have therefore not recorded these figures in detail.

The sugars investigated were lactose, glucose, fructose, sucrose and maltose.

Lactose. Of these the only sugar which was not fermented by the yeast was lactose and it alone appeared to exercise no influence on the storage of either carbohydrate or fat within the yeast cell. A 5 % solution of lactose

behaved very much like water; yeast when incubated in it for 45 hours at 25° showed a small storage of fat and a considerable loss of carbohydrate if the solution had been oxygenated and no storage of fat and rather less loss of carbohydrate in the yeast from the non-oxygenated medium.

Glucose, fructose and sucrose. These three sugars closely resembled each other in their influence on the storage of both carbohydrate and fat. After

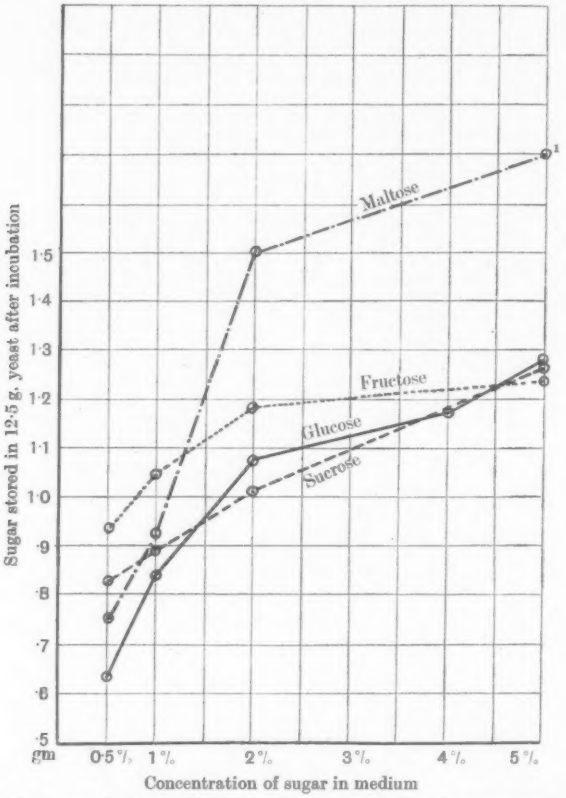

Fig. 2. Curves showing relation of concentration of sugar in non-oxygenated medium to amount of carbohydrate stored by yeast.

incubating a fixed quantity of yeast in a sugar solution the amount of carbohydrate stored depended chiefly on the concentration of sugar in the solution in which the yeast had been incubated. The divergences between the three sugars were most apparent in the very dilute solutions; it was here that the amount of carbohydrate present in the original sample of yeast appeared to exercise some influence, for if equal weights of a yeast with a high and a yeast

[1] The mean of three experiments gave 1·6 g. maltose; as one of these values was abnormally low the number 1·7 has been used in the diagram (cp. Table II).

with a low carbohydrate content were incubated in a 0·5 % or in a 1 % solution of the same sugar, the final carbohydrate content was generally higher in

Curves showing relation between increase of fat after incubation in sugar solutions and concentration of solution.

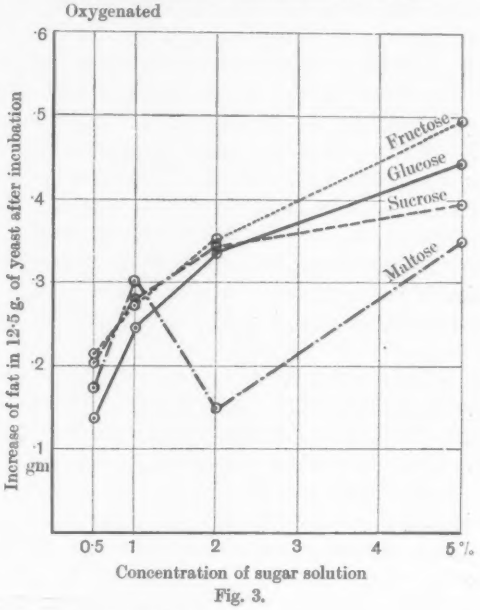

Fig. 3.

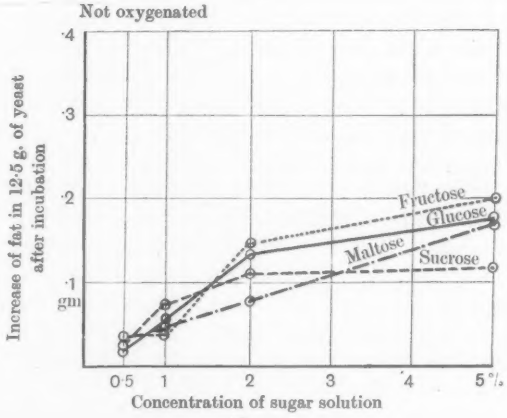

Fig. 4.

the yeast which had the greater content originally. But even with very considerable differences in the carbohydrate contents of the original samples

of yeast, the final amounts of carbohydrate present fell within comparatively narrow limits for any given concentration of sugar.

As the concentration of the solution was increased from 0·5 to 5·0 % the amount of stored carbohydrate rose more sharply at first from 0·5 to 2 %, more gradually from 2 to 5 %. This is apparent from a survey of the curves shown in Figs. 1 and 2.

Fructose in concentrations of from 0·5 to 2 % seems to produce a rather greater storage of carbohydrate than either glucose or sucrose. On the whole we are inclined to think that the figures obtained may possibly represent a real difference in the action of these sugars, though the difference is a small one; the carbohydrate content of yeast after incubation in fructose solutions is higher than that of yeast incubated in glucose and sucrose solutions both with and without oxygenation; and in both cases the difference disappears when the concentration of the sugar rises to 5 %. The effect of oxygenating the sugar solution in which the yeast is incubated is slightly to reduce the carbohydrate content. This result is not invariable but occurs in the majority of cases.

The increase of fat stored up after incubation in an oxygenated solution of glucose, fructose or sucrose is practically the same (Figs. 3 and 4). Fructose gives slightly more fat, but the differences are so small that it is difficult to say whether any significance should be attached to them. The fat-forming power of yeast seems to vary considerably in the different samples and the numbers denoting the amounts of fat stored for any given concentration of sugar in the medium are not as constant as the corresponding numbers denoting the amounts of carbohydrate. For instance four or five experiments were carried out with 5 % solutions of the three sugars; the mean value for the fat content of the yeast after incubation in the fructose solution (oxygenated) was quite appreciably higher than that from the 5 % oxygenated solutions of either glucose or sucrose, but the highest value in any of the experiments was in one of the glucose solutions. We think therefore that though the numbers obtained are suggestive we cannot say with certainty that there is any pronounced difference in the fat-forming powers of these three sugars. With all three the amount of fat deposited in the yeast which has been incubated in a solution of the sugar varies with the concentration of sugar in the medium; there is a gradual flattening in the curve showing the relation of the increase of fat stored to the concentration of sugar, the rate of increase of the fat falling off as the concentration of sugar rises.

The effect of oxygenating the solution is very marked; after oxygenation the fat content is about double as much as in a yeast grown in a similar solution without oxygenation.

The most marked difference between the conditions affecting the storage of fat and of carbohydrate is that, while oxygenation of the solution in which the yeast is incubated diminishes very slightly the amount of carbohydrate in the yeast, it largely increases the formation of fat.

Maltose. Comparison of maltose with the sugars whose action has already been described shows that solutions of maltose of 1 % and over produce markedly greater storage of carbohydrate than do solutions of fructose, glucose or sucrose of similar strength.

Rose [1910] working with *Endomyces vernalis* showed that maltose was a better source of carbon than glucose in a medium which contained also magnesium sulphate, potassium phosphate and asparagine. Lindner and Saito [1910] tested the assimilating power of a very large number of yeasts for various sugars and came to the conclusion that maltose was the best assimilated; Kita's [1914] experiments also confirm this view. Kluyver [1913], starting from the standpoint that these results are not in accordance with the view that all substances before absorption are broken up into the simplest "bricks," repeated Lindner's experiments weighing his yeast after drying at 105° and found definite increases when Kahlbaum's maltose was used, smaller or no increases when the maltose had been first purified by recrystallisation from 80 % alcohol. He found also that the original Kahlbaum sample of maltose contained 0·22 % nitrogen which was reduced after purification to only 0·04 %; he therefore argued that the asparagine of the original solution provided an insufficient nitrogen supply and the effect of the maltose was due to the nitrogenous impurities it contained. Our experiments differed from Lindner's in that our media contained no nitrogen and we measured directly the carbohydrate stored; determinations of the nitrogen in the yeast samples were made both before and after the incubation, and within the limits of the experimental error the nitrogen content was unchanged. The nitrogen contents of the sucrose and maltose used in these experiments were identical. The experiments now carried out seem to us to show quite conclusively that *when yeast is incubated in maltose solutions of from* 1 *to* 5 % *there is much greater carbohydrate storage in the yeast cells than when solutions of glucose, fructose or sucrose are used.*

Maltose solutions however show themselves on the whole to be rather less effective in producing an increase of fat than are solutions of either glucose, fructose or sucrose.

Another point observed while carrying out these experiments is perhaps worthy of notice. After yeast had been incubated in 2 % and in 5 % sucrose solution the weight of the pressed yeast was very much smaller than in the case of yeast from the corresponding solutions of any other sugar. The yeast seemed to have undergone a certain amount of plasmolysis, the dry weight of the yeast, and the amounts of protein, carbohydrate and fat being the same, but the weight of the pressed yeast being only about two-thirds that of the yeast after incubation in solutions of the other sugars.

As the different samples of yeast showed much variation in their power of forming fat, equal quantities of the same sample of yeast were incubated at the same time under similar conditions in a 2 % solution of glucose and in a 2 % solution of one of the sugars, fructose, sucrose or maltose, so that a direct

Table II.

Sugar %	Temp. °C.	Age of yeast. Hours	Original yeast			Incubated in oxygenated solution			Incubated in non-oxygenated solution		
			Fat g.	Carbohydrate g.	Protein g.	Fat g.	Carbohydrate g.	Protein g.	Fat g.	Carbohydrate g.	Protein g.
Glucose:											
0·5	25·5	84	0·1057	1·00	1·36	0·1810	0·55	1·27	0·1262	0·62	1·19
,,	Lab.	110	0·1017	1·00	1·39	0·1667	0·57	1·26	0·1040	0·65	1·40
,,	24·5	109	0·1012	0·65	1·46	0·2720	0·41	1·25	0·1231	0·42	1·37
,,	25	106	0·1040	1·02	1·23	0·1556	0·50	1·12	0·1318	0·66	1·16
,,	25	85	0·0646	1·12	1·24	0·3660	0·79	1·15	0·0842	0·78	1·15
1·0	25	110	0·1051	0·82	1·31	0·3358	0·92	1·35	0·1413	0·75	1·28
,,	25·5	89	0·1041	1·18	1·26	0·4618	0·83	1·19	0·1830	0·90	1·21
,,	24	85	0·0880	1·06	1·14	0·2432	0·71	1·15	0·1457	0·86	1·13
2·0	25·5	89	0·0976	1·07	1·34	0·4220	0·96	1·17	0·2326	0·80	1·27
,,	25·5	84	0·1041	1·47	1·18	0·4805	0·84	1·15	0·2350	0·99	1·09
,,	25	—	0·0905	1·37	1·15	0·3610	1·03	1·06	0·2220	1·27	1·08
,,	25·5	105	0·0805	1·18	1·18	0·3703	0·98	1·17	0·2074	1·22	1·16
,,	25	81	0·0785	1·53	1·16	0·4770	1·04	1·11	0·2096	1·04	1·07
,,	25	103	0·0890	0·83	1·26	0·4818	0·84	1·24	0·2123	1·17	1·18
,,	25	84	—	—	—	0·5095	0·98	1·20	0·2498	0·98	1·20
5·0	27·5	106	0·0985	0·70	1·33	0·5286	1·18	1·31	0·2645	1·20	1·27
,,	25·5	84	0·1055	0·69	1·44	0·3956	1·37	1·35	0·2190	1·40	1·29
,,	25·5	84	0·9870	1·65	1·13	0·7110	1·08	1·00	0·3495	1·24	0·99
Fructose:											
0·5	25·5	86	0·1018	1·52	1·22	0·1853	0·99	1·19	0·1306	1·01	1·22
,,	25	76	0·1042	1·18	1·22	0·4015	0·88	1·21	0·1355	0·99	1·14
,,	24·5	84	0·0800	1·46	1·19	0·3477	1·09	1·15	0·1183	1·08	1·15
,,	25	88	0·0677	1·03	1·37	0·2380	0·77	1·26	0·1077	0·80	1·26
,,	25	85	0·0646	1·12	1·24	0·3404	0·77	1·19	0·0993	0·78	1·21
1·0	25	82	0·1056	1·13	1·22	0·3222	0·85	1·16	0·1248	1·00	1·17
,,	25·5	—	0·0925	1·17	1·26	0·4245	1·21	1·23	0·1414	1·08	1·14
2·0	24·5	82	0·1037	1·09	1·31	0·3658	0·97	1·20	0·2327	1·22	1·13
,,	24·5	82	0·1037	1·09	1·31	0·4258	1·00	1·25	0·2066	1·22	1·10
,,	25	83	0·0885	1·26	1·24	0·5465	1·08	1·14	0·2574	1·18	1·10
,,	25	82	0·1056	1·13	1·22	0·4956	0·91	1·17	0·2305	1·08	1·15
,,	25·5	—	0·0890	0·83	1·28	0·4729	0·93	1·23	0·3007	1·12	1·23
,,	25	80	0·0848	1·45	1·18	0·3794	1·12	1·13	0·2395	1·34	1·17
5·0	25·5	86	0·0979	1·00	1·28	0·5575	1·13	1·49	0·2574	1·24	1·45
,,	,,	118	0·0977	0·95	1·10	0·6266	1·35	1·07	0·3358	1·35	1·01
,,	,,	96	0·1046	0·82	1·43	0·5360	1·29	1·37	0·2254	1·10	1·36
Sucrose:											
0·5	25	84	0·0941	1·31	1·23	0·3457	0·68	1·14	0·1252	0·80	1·22
,,	,,	87	0·0910	1·24	1·25	0·2874	0·77	1·18	0·1226	0·87	1·15
,,	,,	82	0·0835	0·84	1·33	0·2516	0·73	1·15	0·0891	0·80	1·17
1·0	,,	106	0·1022	1·22	1·29	0·2810	0·96	1·21	0·1462	0·99	1·20
,,	,,	86	0·0970	1·47	1·20	0·4405	0·71	1·18	0·1927	0·86	1·15
,,	,,	91	0·0706	0·77	1·37	0·3860	0·83	1·22	0·1457	0·82	1·23
2·0	,,	106	0·1050	1·13	1·30	0·4355	1·03	1·27	0·2107	1·12	1·23
,,	,,	81	0·0785	1·53	1·16	0·4704	0·80	1·09	0·1996	0·93	1·09
,,	,,	84	0·0943	1·12	1·23	0·3980	0·90	1·16	0·2012	0·99	1·16
5·0	,,	84	0·0815	1·23	1·25	0·3803	1·18	1·13	0·2037	1·40	1·15
,,	,,	85	0·0998	1·25	1·30	0·3500	1·30	1·16	0·1750	1·40	1·15
,,	,,	106	0·0928	1·00	1·24	0·6008	1·22	1·10	0·2522	1·18	1·10
,,	,,	96	0·0888	1·10	1·28	0·6097	1·07	1·18	0·2398	1·06	1·12
Maltose:											
0·5	25·5	87	0·1040	1·00	1·30	0·3041	0·71	1·24	0·1350	0·77	1·25
,,	24·5	85	0·0867	1·02	1·32	0·2433	0·72	1·30	0·0935	0·72	1·21
1·0	25·5	110	0·1001	1·00	1·25	0·2978	1·19	1·26	0·1591	1·17	1·17
,,	24·5	96	0·0673	0·72	1·38	0·4919	0·79	1·33	0·0972	0·68	1·27
2·0	25	84	0·0870	0·86	1·18	0·2134	1·31	1·09	0·1781	1·55	1·23
,,	25·5	105	0·0805	1·18	1·18	0·2962	1·33	1·27	0·1880	1·61	1·18
,,	24	108	0·0878	0·93	1·33	0·1863	0·98	1·27	0·1238	1·33	1·24
5·0	26	104	0·1045	1·05	1·30	0·4205	1·82	1·21	0·2760	1·71	1·18
,,	25	118	0·0977	0·95	1·10	0·4697	1·95	1·02	0·3165	1·80	1·05
,,	25·5	85	0·0945	1·29	1·25	0·4639	1·76	1·23	0·2328	1·30	1·14

comparison might be made. The fat contents of the yeasts incubated in the oxygenated solutions of glucose, fructose and sucrose were found to agree within limits of experimental error, but there was appreciably less fat in the yeast incubated in the maltose solution.

Table III. *Showing increase of fat and carbohydrate produced by incubating equal quantities of the same samples of yeast in 2 % solutions of two different sugars.*

			Original yeast		Incubated in oxygenated solution		Incubated in non-oxygenated solution	
Sugar 2 %	Temp. ° C.	Age of yeast. Hours	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.
Glucose	25	103	0·0890	0·83	0·4818	0·84	0·2123	1·17
Fructose	,,	—	—	—	0·4729	0·93	0·3007	1·12
Glucose	,,	81	0·0785	1·53	0·4770	1·04	0·2096	1·04
Sucrose	,,	—	—	—	0·4704	0·80	0·1996	0·93
Glucose	,,	105	0·0805	1·18	0·3703	0·98	0·2074	1·22
Maltose	,,	—	—	—	0·2962	1·33	0·1880	1·61

THE PART PLAYED BY OXYGEN IN PRODUCING AN INCREASE OF FAT.

In order to be sure that the effect of the oxygen was not merely the mechanical one of removing the carbon dioxide, such as that described by Slator [1921], we replaced the current of oxygen by one of hydrogen when incubating the yeast in a 5 % solution of glucose. The control in which no gas was passed gave figures similar to those obtained when a current of hydrogen was passed through the solution. It follows therefore that the effect of the oxygen is not a mechanical one but is specific to oxygen.

PART III. THE INFLUENCE OF PHOSPHATES.

The formation of hexosephosphate when sugars are fermented by yeast juice in the presence of phosphates was first established by Harden and Young [1905, 1908, 1909]; this compound appears to be fructose diphosphate and it is formed when either glucose, mannose or fructose undergoes fermentation. Whereas Harden and Young regard the formation of this compound as an essential reaction in the fermentation of sugar brought about by the living yeast cell, Neuberg, Levite and Schwenck regard its formation as a pathological phenomenon [1917]. Since its discovery a large amount of work has been carried out on the constitution and properties of this substance and evidence has been brought forward suggesting that the presence of a hexosephosphate may not be confined to yeast juice [Embden and Lacquer, 1921]. The probable presence of this substance in muscle [Embden and Lacquer, 1914, 1921] and the detection of an enzyme acting on it in ossifying cartilage [Robison, 1923] are also observations of great interest.

It is well known that the radicals of phosphoric acid and of the fatty acids are associated together in the lipins; the wide distribution of these compounds throughout both the animal and vegetable kingdoms has given rise to much speculation as to their function in the metabolism of the cell.

They have been regarded as the form in which fat is transported in the body, and evidence has been brought forward that in a lactating animal the phosphatide content of the blood in the mammary vein is less than that in the jugular vein [Meigs, Blatherwick and Cary, 1919, 1920]: from this the conclusion has been drawn that the phosphatides of the blood are utilised by the mammary gland to form milk fat. In spite however of the large number of investigations which have been carried out, the function of the lipins and the rôle they play in the life of the cell cannot be said to have been satisfactorily elucidated.

The fact that the hexose molecule and the fatty acid radicals are each found in association with the phosphate radical suggests that a more complete knowledge of the phosphorus metabolism of the cell may throw some light on the processes by which fat is manufactured from carbohydrate in the living organism. We endeavoured therefore to find out whether the formation of fat in the yeast cell was in any way influenced by the presence of phosphates in the solutions in which the yeast was incubated.

The first question investigated was whether the conditions under which the yeast was incubated could be so modified as to produce an increased storage of phosphate in the yeast cell. We therefore examined the effect of changes in the composition of the medium in which the yeast was incubated and determined the effect on the total phosphorus content of the yeast produced by the addition of sugar to the phosphate solution in which the yeast was incubated.

The method adopted was a follows: 12·5 g. of pressed yeast which had been well washed were incubated for 45 hours at 25° in a litre of a solution of sugar containing a mixture of 0·3962 % Na_2HPO_4 and 0·0286 % KH_2PO_4, a mixture giving a p_H of about 7·8. The sugar to be investigated was then added in varying concentrations, the percentage of phosphate being kept constant throughout the series of experiments. At the end of 45 hours the yeast was filtered off, washed until the washings gave no reaction for phosphate and the total amount of phosphorus it contained determined by Neumann's method. An analysis of 12·5 g. of the original sample of yeast was also made so that the increase of phosphorus could be calculated. The fat and carbohydrate were estimated as previously described [Smedley MacLean, 1922] after hydrolysing the yeast by boiling for two hours with normal acid; the protein content of the yeast was also determined.

It was found that the increased amount of phosphate taken up by the yeast cell depended on the concentration of sugar in the solution in which the yeast had been incubated.

The effect of the three sugars, glucose, fructose and sucrose, was practically identical. The amount of phosphate taken up from the solution by the yeast cell varied with the concentration of sugar, increasing as the proportion of sugar in the medium rose from 0·5 to 5·0 %. The action of maltose also produced an increase in the phosphorus content of the yeast after incubation,

the increase being dependent on the strength of the maltose solution in which the yeast was incubated. The increase of phosphate in the yeast was however appreciably less when maltose was substituted for the other sugars mentioned, the difference being most marked in the stronger solutions.

The actual numbers obtained are shown in Table IV and the relation of the increase of phosphate to the concentration of the sugar solution is expressed by the curves shown in Figs. 5 and 6.

Table IV. *Showing amount of phosphorus in* 12·5 *g. of pressed yeast after being incubated for* 45 *hours in a litre of sugar solution containing* 0·3962 % Na_2HPO_4 + 0·0286 % KH_2PO_4.

	Amount of P in sample				Amount of P in sample		
Conc. of sugar %	(1) Original g.	(2) After incubation in oxygenated solution g.	(3) After incubation without oxygenation g.	Conc. of sugar %	(1) Original g.	(2) After incubation in oxygenated solution g.	(3) After incubation without oxygenation g.
Glucose:				Fructose:			
0·5	0·0613	0·0687	0·0663	0·5	0·0561	0·0777	0·0568
	0·0617	0·0689	—		0·0583	—	0·0586
1	0·0618	0·0754	0·0734		0·0679	0·0716	0·0656
	0·0642	0·0764	0·0731		0·0673	0·0713	0·0650
	0·0602	0·0865	0·0893	1	0·056	0·067	0·0697
	0·0598	0·0850	0·0870		0·057	0·062	—
2	0·0611	0·0860	0·0786	2	0·0682	0·1012	0·0900
	0·0586	0·0870	—		0·0718	0·1023	0·0921
	0·0638	—	0·0740		0·0598	0·0880	0·0747
	—	—	0·0730		0·0566	0·0879	0·0757
					0·0611	0·0860	0·0761
4	0·0650	0·1050	0·1024		0·0586	0·0846	0·0742
	0·0633	0·0989	0·0882				
5	0·0607	0·1021	0·1035				
	0·0609	—	0·1124	5	0·077	0·128	0·102
	0·0580	0·1252	0·1158		0·065	0·123	0·098
	0·0586	0·1310	0·1140				
	0·0694	0·1023	0·0962				
	0·0694	0·1009	0·0919				
Maltose:				Sucrose:			
0·5	0·0640	0·0566	0·0681	0·5	0·0606	0·0668	0·0639
	0·0637	0·0510	0·0653		0·0578	0·0749	0·0634
1	0·0634	0·0770	0·0602	1	0·0620	0·0706	0·0632
	0·0630	0·0706	0·0611		0·0618	0·0717	0·0676
2	0·0600	0·0776	0·0700	2	0·0708	0·0934	0·0839
	0·0594	—	0·0714		0·0742	0·0965	0·0799
5	0·0656	0·0949	0·0767	5	0·0596	0·1102	0·0883
	0·0684	—	0·0757		0·0603	0·1105	0·0881
	0·0709	0·0903	0·0772				
	0·0679	0·0898	0·0780				

It is of course possible that the numbers obtained at the end of the experiment represent a balance; that for instance hexosephosphate is being continually formed and decomposed and that in the dilute sugar solutions the figures are low because there is insufficient sugar to reform the hexosephosphate which has been decomposed. The residual sugar was always determined at the end of each experiment; it was generally found that in the

0·5 % solutions all the sugar had been used up, but in the higher concentrations some sugar always remained.

The effect of oxygenation on the amount of phosphate taken up by the yeast cell was found to be quite marked. Oxygenation of the solution increased considerably the amount of phosphate stored by the yeast cell.

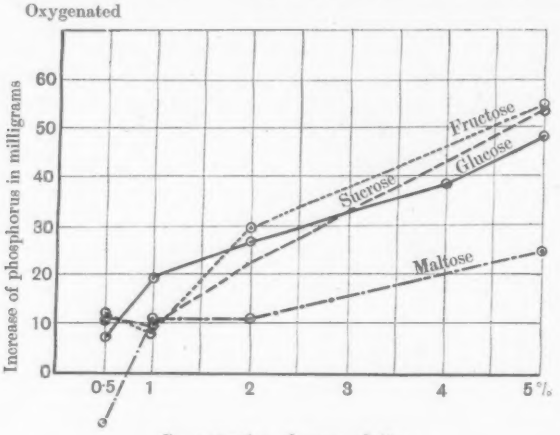

Fig. 5. Curves showing relation between concentration of sugar in solution and increase of phosphorus in yeast.

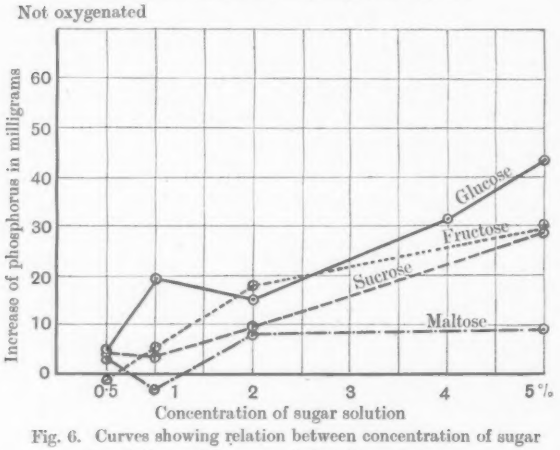

Fig. 6. Curves showing relation between concentration of sugar in solution and increase of phosphorus in yeast.

The above results indicate that phosphate is taken up by the yeast cell from the medium in which it is incubated, in association with sugar and strongly support Harden's view that hexosephosphate is a normal stage in the metabolism of the cell.

The second point investigated was whether the addition of phosphate to the sugar solution in which the yeast was incubated had any influence on the amount of fat or carbohydrate contained in the yeast.

The method by which this was determined was to incubate quantities of 12·5 g. of the same sample of yeast under precisely similar conditions in a litre of sterilised sugar solution with and without the addition of phosphate and to analyse the sample of yeast before and after it had been incubated. Four Winchester quart bottles were placed in the incubator at the same time; two contained a litre of the sugar solution which it was desired to investigate, and in each of the other two was placed a litre of the sugar solution to which the phosphate mixture had been added in the proportions above described.

The solution in one of each pair of bottles was oxygenated throughout the experiment so that the effect of oxygenating the solution could at the same time be determined.

Influence of the addition of phosphates on the formation of fat.

The results of these experiments show that the presence of phosphates exerts a quite considerable effect in increasing the storage of fat if the solution in which the yeast is incubated is oxygenated during the experiment. If the solution be not so oxygenated the result is less marked, but in the majority of cases even when the medium is not oxygenated the yeast in the phosphate-containing solution has the higher fat content. Very dilute solutions of fructose were rather variable in their behaviour, but with stronger solutions the yeast always showed an increased fat content after incubation. The differences in the increase of fat produced by the addition of phosphates to the oxygenated solutions of the different sugars examined were of the same order; no differences which could be regarded as significant were observed between the effects of maltose, glucose, fructose or sucrose.

Analysis of the yeast samples before and after incubation clearly indicated that the addition of phosphates to the oxygenated sugar solutions produced an increased fat content of the yeast; we were however unable to establish a relation between the amount of increase of fat produced by the addition of phosphates to the oxygenated solution and the concentration of sugar in the medium.

Influence of the addition of phosphates on the carbohydrate content.

We have shown that oxygenation of the sugar solution in which the yeast is incubated on the whole tends to diminish the carbohydrate content of the yeast when compared with yeast grown in a similar solution without oxygenation; this effect is however not invariable and in most cases was found to be slight.

The effect of the addition of phosphates to the sugar solution was somewhat to increase the difference between the carbohydrate content of the

Table V. *Showing the effect of incubating yeast in solutions of 0·4 % alkaline phosphate with different concentrations of sugar. Influence on fat and carbohydrate content.*

%	Temp. °C.	Age of yeast. Hours	(1) Original sample of 12·5 g. pressed yeast			(2) After incubation in sugar solution oxygenated			(3) After incubation in sugar solution not oxygenated			(4) After incubation in sugar solution + phosphate solution oxygenated			(5) After incubation in sugar solution + phosphate solution not oxygenated		
			Fat g.	Carbohydrate g.	Protein g.	Fat g.	Carbohydrate g.	Protein g.	Fat g.	Carbohydrate g.	Protein g.	Fat g.	Carbohydrate g.	Protein g.	Fat g.	Carbohydrate g.	Protein g.
Glucose:																	
0·5	25	106	0·1040	1·02	1·23	0·1556	0·50	1·12	0·1318	0·66	1·16	0·2257	0·63	1·14	0·1198	0·75	1·07
1	24	85	0·0880	1·06	1·14	0·2432	0·71	1·15	0·1457	0·86	1·13	0·4335	0·70	1·16	0·1666	0·99	1·16
2	25	—	0·0905	1·37	1·15	0·3610	1·03	1·06	0·2220	1·27	1·09	0·4340	0·78	1·07	0·2580	1·02	1·09
	25	94	0·0854	1·17	1·27	—	—	—	—	—	—	0·5389	0·98	1·19	0·2615	0·99	1·17
4	25	79	0·0772	0·89	1·27	0·2690	1·03	1·19	0·2470	1·17	1·20	0·4528	0·91	1·24	0·2391	1·02	1·22
5	25·5	87	0·1029	0·81	1·30	0·3215	1·18	1·27	0·2360	1·26	1·19	0·4522	0·87	1·22	0·2544	1·06	1·16
	25·5	109	0·1102	1·02	1·26	—	—	—	—	—	—	0·9012	1·26	1·16	0·4542	1·62	1·11
Fructose:																	
0·5	24·5	84	0·0800	1·46	1·19	0·3477	1·09	1·15	0·1183	1·08	1·15	0·3089	0·91	1·15	0·1089	1·13	1·15
	25	88	0·0677	1·03	1·37	0·2380	0·77	1·26	0·1077	0·80	1·26	0·3231	0·75	1·24	0·1270	0·74	1·25
1	25·5	—	0·0925	1·17	1·26	0·4245	1·21	1·23	0·1414	1·08	1·14	0·3304	0·87	1·30	0·1464	0·92	1·26
2	25	83	0·0885	1·26	1·24	0·5465	1·08	1·14	0·2574	1·18	1·10	0·5495	1·05	1·18	0·1822	1·36	1·12
		80	0·0848	1·45	1·18	0·3794	1·12	1·13	0·2395	1·34	1·17	0·5550	1·06	1·17	0·2524	1·19	1·14
		94	0·0854	1·17	1·27	—	—	—	—	—	—	0·6167	1·06	1·18	0·2615	1·28	1·18
5	25·5	—	0·1046	0·82	1·43	0·5360	1·29	1·37	0·2254	1·10	1·36	0·8180	1·25	1·33	0·3680	1·63	1·35
Sucrose:																	
0·5	25	82	0·0835	0·84	1·33	0·2516	0·73	1·15	0·0891	0·80	1·17	0·3334	0·72	1·17	0·1242	0·79	1·01
1	27·5	91	0·0706	0·77	1·37	0·3860	0·83	1·22	0·1457	0·82	1·23	0·3562	0·84	1·24	0·1277	0·83	—
2	25	84	0·0943	1·12	1·23	0·3980	0·90	1·16	0·2012	0·99	1·16	0·5441	0·94	1·13	0·2021	1·12	1·14
5	25	96	0·0888	1·10	1·28	0·6097	1·07	1·18	0·2398	1·06	1·12	0·7390	0·94	1·15	0·2669	1·17	1·15
Maltose:																	
0·5	24·5	85	0·0867	1·02	1·32	0·2433	0·72	1·30	0·0935	0·72	1·21	0·3190	0·71	1·23	0·0818	0·46	1·25
1	24·5	96	0·0673	0·72	1·38	0·4919	0·79	1·33	0·0972	0·68	1·27	0·4960	0·81	1·29	0·1128	0·58	1·31
2	25	84	0·0870	0·86	1·18	0·2134	1·31	1·09	0·1781	1·55	1·23	0·3552	1·14	1·06	0·2083	1·22	1·16
	24	108	0·0878	0·93	1·33	0·1863	0·98	1·27	0·1238	1·33	1·24	0·3454	0·70	1·26	0·1411	0·92	1·26
5	25·5	85	0·0945	1·29	1·25	0·4639	1·76	1·23	0·2328	1·30	1·14	0·5608	1·62	1·17	0·2335	1·48	1·03
	25	96	0·1068	0·90	1·33	—	—	—	—	—	—	0·4907	1·48	1·25	0·2309	1·89	1·26

yeast incubated in the sugar solution with and without the passage of a current of oxygen. The carbohydrate content of the yeast incubated in the non-oxygenated solution was the greater and the addition of phosphate to the sugar solutions which were oxygenated during the period of incubation led to a distinct diminution in the carbohydrate content of the yeast.

Influence of the addition of phosphates on the protein content.

The differences in the protein content of the yeast before and after incubation in the sugar solutions to which phosphates had been added were very small; and considering the difficulties of carrying out these experiments without any loss of yeast during filtration, they cannot be regarded as outside the range of experimental error.

Summary and discussion of results.

(1) When yeast was incubated in oxygenated water, part of the carbohydrate originally present disappeared and an increase of fat took place. The addition of propyl, butyl and *iso*-amyl alcohols in decimolar concentration exerted an inhibitory effect; decimolar solutions of the sodium salts of formic, propionic and butyric acids, glycol, glycerol and acetone behaved like water.

(2) When yeast was incubated in oxygenated 0·5 % solutions of ethyl alcohol or of the sodium salts of acetic, lactic or pyruvic acids, an effect was exerted on the fat content of the yeast similar to that produced by 0·5 % solution of glucose. Increasing the concentration of these substances in the solutions did not however lead to further increase of the fat content of the yeast, as in the case of the sugars.

A possible explanation is that these simpler molecules may be used to build up carbohydrate and only indirectly lead to the production of fat; this suggestion is supported by the observation that in certain cases where the yeast was incubated in oxygenated solutions of ethyl alcohol and of sodium acetate an increase in the total carbohydrate of the yeast cell was observed.

(3) When yeast was incubated under the conditions described in a solution of fructose, glucose or sucrose, at the end of 45 hours both fat and carbohydrate were found to have been stored up by the yeast cells. The amounts stored up depend on the concentration of sugar in the medium and are independent of the nature of the sugar; the rate of increase of the carbohydrate content diminishes as the concentration of the sugar in the medium rises; the rate of increase of the fat content also falls, but much more than that of the carbohydrate content, as the sugar concentration increases.

(4) Maltose differs markedly in its behaviour from the three sugars, glucose, fructose and sucrose. It is more potent in producing a storage of carbohydrate and rather less effective in building up fat.

The difference in the power of storing carbohydrate is so marked that it seems impossible that the maltose can be split into glucose before assimilation, and we must conclude that maltose is directly dealt with as such by the yeast

cell and there built up into reserve carbohydrate. It is interesting to recall that the recent work of Irvine [1923] points to the presence of a maltose unit in the glycogen molecule; this conclusion is in accordance with the behaviour of the yeast cell which stores carbohydrate more readily when fed with a solution of maltose than with one of glucose.

The storage of fat after yeast has been incubated in maltose solutions was rather less marked than with the other sugars; in 0·5 and 1 % oxygenated solutions as much fat was formed by the yeast as when it was incubated in the corresponding solutions of glucose, fructose or sucrose, but after incubation in a 2 or 5 % maltose solution less fat was formed. No increase in fat content corresponding with the increased carbohydrate content was observed.

(5) Oxygenation of the medium throughout the period of incubation made a very great difference to the amount of fat produced, but made little difference to the final carbohydrate content, generally producing a slight diminution in amount.

(6) The addition of phosphates to the oxygenated sugar solutions produced an increase in the amount of fat stored and a diminution in the amount of carbohydrate. If the solutions were not oxygenated, the increase of fat was small or absent.

(7) If about 0·4 % of alkali phosphates was added to solutions of the different sugars in which the samples of yeast were incubated, phosphate was taken up by the yeast cell in proportion to the concentration of sugar in the solution in which the yeast was incubated. The amount of phosphate was greater in the yeast incubated in the oxygenated solutions than in that incubated in solutions which were not oxygenated.

These results seem best explained by the hypothesis that carbohydrate storage in yeast takes place in two ways: (1) as glycogen or some other similar compound giving a reducing sugar on hydrolysis and (2) as a hexosephosphate which forms the first stage in the transformation of carbohydrate to fat.

The view that carbohydrate is absorbed and combined with phosphate forming a hexosephosphate is supported by the work of Harden and Young on the isolation of hexosephosphates from yeast juice and by the evidence now brought forward that the phosphate content of the yeast depends on the concentration of the sugar in the medium in which the yeast is incubated.

The second part of the hypothesis now put forward that the formation of a hexosephosphate may be regarded as the first stage in the transformation of carbohydrate to fat is supported by the fact that the addition of phosphates to the solution of sugar in which the yeast is incubated somewhat increases the fat content of the yeast if the solution be oxygenated during the period of incubation.

Although the proportion of oxygen is much less in the fat than in the carbohydrate molecule, the part played by the oxygen is evidently a very important one in the transformation of carbohydrate to fat. Oxygenation of

the sugar solution in which yeast is incubated produces a yeast with more than double the fat content of a yeast incubated in a sugar solution which has not been so oxygenated; and if additional phosphate be added to the medium the fat content is still further raised if the solution continues to be oxygenated.

The increase of fat stored up when phosphate is added to the oxygenated sugar solution in which the yeast is incubated seems to us to be best explained by assuming that oxidation of a hexosephosphate or of some material derived from it forms an essential stage in the story of the fat metabolism of yeast.

It is possible that simpler molecules such as those of pyruvic acid or aldehyde may be formed at a later stage, or it is possible that groups of this nature may be associated in some complex compound containing the phosphate radical. At present there is no evidence which throws light on this question and it remains only a matter of speculation.

We desire to express our thanks to Mr J. L. Baker and to Messrs Watney, Coombe, Reid and Co. for supplying us with yeast grown under approximately constant conditions and to acknowledge our indebtedness to the Food Investigation Board of the Department of Scientific and Industrial Research for giving us the opportunity of carrying out this work.

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XC. STUDIES IN BACTERIAL NUTRITION. I.

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In approaching the study of bacterial nutrition there are two main lines of attack. First there is what may be called the analytical method, under which an attempt is made to separate a medium into its component substances or groups of chemically similar individuals, and then to determine which of these are essential to the life of the organism. Exceedingly little work has been done from this point of view, because no doubt of the lack of satisfactory methods for analysing proteins and their derivatives, and the difficulty of avoiding the introduction of bactericidal substances during chemical manipulation. The most recent work on these lines is that of Mueller [1922], who claims to have isolated a new amino acid, which he states to be one of the substances essential for the growth of the streptococcus.

The second method is the deductive one, which consists in following the changes occurring in the nitrogenous constituents of the medium during the growth of the organism, and thence attempting to elaborate a theory of its metabolism. A fair amount of attention has been devoted to this side of the question. Sears [1916] gives a comprehensive account of the progress so far made, and De Bord [1923] brings the summary up to date. The difficulty, however, of determining what relation the variation in amino acids, ammonia, and other end products, bears to the life history of the micro-organism, and in distinguishing between primary and secondary reactions in the medium, leaves the whole question as yet unsolved.

The object of the present research was to determine the growth substances for one organism in a given medium, by applying some of the chemical methods used for protein analysis. The medium was a pancreatic digest of caseinogen, first devised by Cole and Onslow [1916], but in the present case the whole pancreas finely minced was used instead of an extract; 100 g. of pancreas to 200 g. of caseinogen in a volume of 2 litres. This medium is used now in this laboratory as a stock medium. It gives with those organisms which will grow on a peptone-lemco medium, a luxuriant growth, which, however, is said rapidly to lose its viability. The use of a phospho-protein like caseinogen results in the finished medium being infinitely better buffered than most peptone media. The following table gives several chemical analyses of typical stock broths.

Analyses of stock broth 1 *in* 3.

% total nitrogen	% amino + ammonia N	% total solids	% ash	% ammonia N	% nitrogen unprecipitable by phospho-tungstic A
0·4110	0·2949	3·590	0·240	0·044	0·3162
0·3714	0·2786	3·044	0·218	0·032	0·2888
0·3267	0·2038	2·792	0·230	0·020	0·1880
0·3879	0·3139	3·370	0·244	0·035	0·2796
0·3671	0·3098	3·074	0·250	0·025	0·2367
0·3578	0·2571	3·070	0·218	0·024	0·2153

The amino acid and ammonia nitrogen were determined by formalin titration in alcoholic solution after the method of Foreman [1920]. This method gives a sharp end point, and is much more satisfactory for a liquid of this type which contains all stages of protein hydrolytic products, than either Sörensen's or Van Slyke's, both of which were tried in the first instance but subsequently discarded.

The organism decided upon was a streptococcus, by reason of its being intermediate in luxuriance between the easily growing coliform bacilli and staphylococcus, and the very delicate gonococcus and meningococcus. One thus has an organism which although reasonably easy to cultivate, seems to require certain specific substances for its growth, unlike bacilli of the coli type which can probably utilise many forms of nitrogen. The strain of streptococcus which was here used as the experimental organism was one which fermented only mannitol and saccharose, and was haemolytic, thus corresponding in Holman's classification to *Streptococcus haemolyticus* III. It was grown throughout on caseinogen digest medium, so that it should be fairly stabilised as regarded its requirements.

The first point proved was the heat stability of the growth substances contained in caseinogen broth.

Into each of 30, $3\frac{1}{2}'' \times \frac{3}{8}''$ tubes in a rack was measured 3 cc. of 1 in 3 stock broth, and after plugging with wool, the tubes were steamed for one hour. On 30 successive days the steaming was repeated, one tube being removed from the rack after each day's steaming; thus the tubes had from one to thirty hours' steaming. At the end of thirty days, when they were sown with a suspension of the streptococcus, no visible difference in growth was observed between the first and last tubes. Thus the growth substances are stable so far as a reasonable amount of steaming goes.

Autoclaving proved to be more destructive. A rack of 18 tubes of 1 in 3 stock broth was autoclaved for 20 minutes at 117° C. on successive days, one tube being removed each day, the procedure being similar to that employed in the steaming experiment. On sowing with the streptococcus, only the first eight tubes showed growth after 24 hours, but at the end of 48 hours, practically equal growth was present in all the tubes. Evidently prolonged autoclaving brings about some change in the broth which delays the growth of the streptococcus.

With regard to the efficiency of the growth substances it was found that growth of the streptococcus could be detected in an average sample of stock broth when diluted 1 in 96, which is equivalent to less than 0·1 of caseinogen.

The first line tried was the separation of caseinogen stock broth into two fractions by means of phosphotungstic acid, in an acid solution, according to Van Slyke's method [1912]. In preliminary experiments the effect of adding inorganic salts during chemical manipulation was very soon realised to be of importance. Rough experiments were carried out to determine their effect on growth, and the following results were obtained with substances liable to be introduced during the precipitation.

Sodium chloride. % NaCl added to stock broth:

	Control *	0·5	1	2	3	4
Streptococcus	+ + + +	+ + + +	+ + + +	+ +	+ +	+
Staphylococcus	+ + + +	+ +	+ +	+ +	+ +	+ +
B. shiga	+ + + +	+ + + +	+ + + +	+ +	±	−
B. coli	+ + + +	+ + + +	+ + + +	+ +	+	+

* The stock broth used as control contained less NaCl than could be estimated by titration with $AgNO_3$ (*i.e.* less than 0·1 %).

Barium chloride. % $BaCl_2$ added to stock broth:

	Control 0·0	0·5	1	2	3	4
Streptococcus	+ + + +	−	−	−	−	−
Staphylococcus	+ + + +	+ +	+	+	−	−
B. shiga	+ + + +	+ + + +	+ +	+ +	±	−
B. coli	+ + + +	+	±	±	−	−

Phosphotungstic acid. % phosphotungstic acid added to stock broth:

	Control 0·0	0·5	1	2	3	4
Streptococcus	+ + + +	+ + + +	+ + + +	+ +	−	−
Staphylococcus	+ + + +	+ + + +	+ +	+	±	−
B. shiga	+ + + +	+ + + +	+ +	±	−	−
B. col	+ + + +	+ + + +	+ + + +	+ +	±	±

It will be noted in the case NaCl, that growth of all four organisms was impaired by a concentration of 2 %. This made it essential that HCl introduced into the broth should subsequently be removed by distillation *in vacuo* and not by neutralisation.

50 cc. of stock broth were treated with 18 cc. of conc. HCl and a solution of 15 g. phosphotungstic acid and the whole made up to 200 cc. The mixture was heated on a boiling water-bath until the precipitate nearly redissolved and then allowed to stand for 48 hours, at the end of which time the precipitate was filtered off through a hardened filter paper, and washed with 200 cc. of an ice-cold solution containing 2·5 % phosphotungstic acid, and 3·5 % HCl. In early experiments the removal of the phosphotungstic acid was effected by treatment with $BaCl_2$ in alkaline solution, but later the more convenient treatment with amyl alcohol-ether mixture (Van Slyke) was used, which avoids not only any considerable dilution, but also the introduction of toxic Ba salts. Both precipitate and filtrate were freed from phosphotungstic acid, evaporated to dryness *in vacuo* (temp. not exceeding 60°), and separately redissolved in distilled water, and adjusted to p_H 7·5. Growth was tested by placing the solutions in small tubes, plugging and sterilising (20 mins.

at 117° in the autoclave), and sowing with drops of suspensions of the streptococcus and other organisms from sterile dropping pipettes. The dilutions of all test solutions were so arranged that they were equivalent to the original 1 in 3 broth, or if this was inconvenient to 1 in 6 broth. Appropriate tubes of unaltered broth were always sown as controls. Growth was estimated by opacity and the following scale used.

+ + + + = Opacity equal to the control.
+ + = Opacity equal to the control when the latter diluted with an equal volume of water.
± = Very slight or no evidence of growth, but growth obtained on subculturing on caseinogen digest.
− = No growth on subculturing.
+ + + and + = Intermediate degrees estimated by comparison with control and diluted control.

In neither of the two portions of broth prepared as above, nor in the two combined, was any growth whatever observed with the streptococcus, but with three other representative organisms tried the average results were as follows:

All solutions ≡ broth 1 in 3:

Organism	Phospho-tungstic precipitate	Phospho-tungstic filtrate	Precipitate + filtrate	Control broth 1 in 3
Streptococcus	−	−	−	+ + + +
Staphylococcus	+	+ +	+ + +	+ + + +
B. shiga	+	+ + +	+ + + +	+ + + +
B. coli	+	+ + +	+ + + +	+ + + +

This served to show that for the last three organisms the growth elements in the broth could practically be reconstituted after chemical manipulation. Neither the precipitate nor the filtrate portion showed any growth inhibition when added to stock broth. It was evident that in carrying out the precipitation with phosphotungstic acid in the usual way some substance or substances, essential to the growth of the streptococcus, had been lost or rendered inactive. This missing growth factor was not to be found in the precipitate of humin, which is always formed when the phosphotungstic precipitate is extracted with amyl alcohol-ether mixture, for this proved to be quite inert as regarded growth of the organism, and it seemed likely that some chemical action accounted for the change in the broth.

Treatment with conc. HCl in the same proportion as used in the precipitation, and evaporation *in vacuo*, did not destroy the growth producing power of the broth. Under this treatment the only alteration which sometimes occurred was a change in the character of the growth, which became less agglutinated. Finally after many experiments varying the conditions of precipitation, it was found that impurity in the phosphotungstic acid accounted for the destruction of growth substances, and that when the crystals (British Drug Houses) were carefully repurified by treatment with conc. HCl and ether [Wu, 1920] the precipitation could be carried out by the usual method, and the broth practically reconstituted as regarded growth of the

streptococcus by adding the fractions together. In one experiment the growth in the reconstituted broth was quite as good as that in the control, but in general the results were as follows, some growth being lost.

	Precipitate (P)	Filtrate (F)	P+F	Control broth 1 in 3
Streptococcus	–	±*	+++	++++

* Apparently negative, but growth obtained on subculturing.

It is suggested that this slight growth in the filtrate portion was caused by the presence of a small amount of the precipitated basic amino acids, peptone bodies and ammonium compounds, the phosphotungstates of which are very slightly soluble, and that in the ideal experiment there would be two fractions of the broth neither of which would alone support growth of the streptococcus, but which would constitute a good medium when added together.

In one experiment in which stock broth was divided into two portions (the washings being included with the filtrate), the effect of adding increasing quantities of filtrate portion to decreasing quantities of precipitate portion was tried with the following interesting results.

	1	2	3	4	5	6	7	8	9	10	11	12
Filtrate cc.	0	0·1	0·2	0·3	0·4	0·5	0·6	0·7	0·8	0·9	1·0	Control
Precipitate cc.	1·0	0·9	0·8	0·7	0·6	0·5	0·4	0·3	0·2	0·1	0	
Water cc.	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	
	–	+++	++++	++++	++++	++++	+++	++	++	+	±	++++

The 6th tube≡broth 1 in 3

This seems to indicate that growth depends on the presence of a sufficient quantity of the precipitate portion, which, however, needs to be activated by the presence of a small amount of the filtrate portion.

It is interesting to note that the substance destroyed by the impure phosphotungstic acid, although essential to the growth of the streptococcus, does not affect the growth of the coliform bacilli or of the staphylococcus. Neither do the latter organisms seem to be at all exacting in their requirements, since their luxuriance depends apparently more upon the total nitrogen content of a given solution than upon the presence or absence of specific substances. There are definite indications on the other hand, that two substances at least are essential for the growth of the streptococcus; furthermore, these substances are reasonably stable and admit of chemical manipulation, so that the problem of their identity should be soluble by chemical means.

The precipitation by phosphotungstic acid promises to be of some use in the isolation of the growth substances of the streptococcus, and it is now being applied in conjunction with Dakin's butyl alcohol extraction in an attempt to fractionate the stock broth in a more detailed manner.

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XCI. THE FLUIDITY AND COAGULATION OF THE BLOOD.

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One of the landmarks in the history of the coagulation of the blood is the observation of Fredericq [1878] that heat coagulation occurs when the blood in the excised jugular of a horse is heated to 55–57°, after the subsidence of the corpuscles. As the fibrinogens of the laboratory prepared by the method of Hammarsten [1879] and of Schmidt [1892] coagulate at 56°, it is commonly believed that they are identical with the precursor of fibrin in the blood. This was not however accepted as conclusive by two prominent physiologists, Wooldridge [1885, 1893] and Michael Foster [1888], each pointing out that fibrin ferment (thrombin) could be injected into the circulation in massive amounts without causing thrombosis, while similar quantities added to solutions of the fibrinogens of the laboratory provoke clotting. An attempted explanation of this anomaly, in the terms of Howell's [1911] views on coagulation, was advanced by Gasser [1917], who suggested that thrombin, when intravenously injected, combines with antithrombin to form an inactive substance termed metathrombin, it being assumed, without any direct evidence, that the introduction of thrombin into the circulation causes the liver to secrete an excess of antithrombin. But Pickering and Hewitt [1922, 2] have shown that massive doses of thrombin can be injected into the circulation of animals deprived of hepatic activity without causing intravascular clotting and these authors have deduced evidence that antithrombins are *post-mortem* products. Thus the lack of coagulant action of massive doses of thrombin in circulating blood is at least suggestive of some essential difference in the condition or composition of the fibrinogens of the laboratory and the precursor of fibrin in the living blood.

The salts employed in the preparation of fibrinogen by Hammarsten's method cannot be regarded as without influence on the structure or condition of the latter material, as Howell [1914, 1] has found it is not possible by this method to obtain solutions of fibrinogen of uniform properties, part of the

[1] The theory of the fluidity and coagulation of the blood enunciated in this paper owes its origin and elaboration solely to J.W.P. but I am in entire agreement with it. The experimental work has been carried out in collaboration, the operative part by D. H. de S., the observations on clotting by both. D. H. de S.

protein being denaturated by repeated precipitation. The method of Schmidt [1892], which involves dilution with excess of distilled water, is not well calculated to preserve the surface conditions of colloidal complexes, and Mellanby [1909] has found that the fibrinogen prepared by this process is always contaminated with prothrombin. Only after prolonged adsorption by barium sulphate were Dale and Walpole [1916] able to disassociate prothrombin from fibrinogen. Accepting these data, we shall write of a fibrinogen-prothrombin complex in blood, rather than of two disunited substances fibrinogen and prothrombin.

Nolf [1921] has shown that fibrinogen dissolved in faintly alkaline isotonic solution and fibrinogen dissolved in the protein residues of the plasma behave differently with regard to precipitation by chloroform. This, he explains, as due to protection afforded by the stable colloids in the protein residue, which restore the condition of the prepared fibrinogen to that of the fibrinogen of circulating blood.

In view of the obvious differences of circulating and shed mammalian plasma, it appeared desirable to enquire into the condition of fibrinogen prior to bleeding, rather than subsequent to that act. To this end experiments were devised: (1) to study the effect of heating the blood in the heart to 56–63°, without exposure to the air; (2) to observe the results of heating intraventricular blood to 56–63°, after disturbance of its surface conditions; (3) to examine the coagulability of the fluid blood remaining in the heart, after the formation of mechanically produced intravascular clots and (4) to enquire into the effects on the coagulability of blood produced by heating it to temperatures less than the coagulation temperature of fibrinogen.

General Technique.

Cats and rabbits were used for the experiments, the former were anaesthetised with A.C.E., the latter with ether. In the case of the cats the animals were pithed under the anaesthetic and artificial respiration was maintained. The great vessels round the heart were then tied by a mass ligature, the heart removed and immediately placed either in isotonic saline or in neutral olive oil. Under these conditions the hearts were heated without admission of air into their interiors for the periods and to the temperatures shown in the protocols.

Control experiments were made with a thermometer inserted, through the aorta, into the left ventricle and ligatured in position, so as to prevent the entrance of fluid from the bath into the cavities of the heart. These showed that four minutes sufficed for the intraventricular temperature to exceed 56°, when the heart was placed in a bath at 60° and that after seven minutes the temperature of the ventricular cavity was that of the bath.

The mechanically produced thrombi were obtained by the introduction of cotton threads into the cavities of the heart *in situ*. The contraction of the living heart muscle prevented haemorrhage from the punctures. By the term

"commencement" of coagulation is implied the first visible departure from fluidity, other than the formation of isolated filaments of minute diameter, by "completion" that coagulation has advanced so far that the vessel could be inverted without spilling.

Each experiment described is representative of a number yielding concordant results.

The heating of blood in excised hearts.

Exp. 1. The heart of a cat was excised and placed immediately in an oil bath at 60°. The temperature of the bath fell to 56° and remained at that temperature for five minutes. The bath was raised to 60° and maintained at that temperature for twelve minutes. When removed from the bath, the heart showed a small clot in the right ventricle, with a considerable amount of fluid blood. This clotted on glass and some of it which remained in contact with the surface of the ventricle developed a large clot. The blood from the right ventricle also gave a precipitate when one-fifth saturated with ammonium sulphate and also when half saturated with sodium chloride. The small amount of blood in the left ventricle was completely fluid and subsequently clotted on glass.

Exp. 2. Cat. The clotting times of blood from carotid before excision of the heart were commencement 5′ 30″ and completion 6′ 20″. The heart was excised and placed in a normal saline bath at 62°. For the next eleven minutes the temperature of the bath varied between 60° and 63°, it was then kept at 63° for five minutes. All the blood in the right ventricle was fluid on shedding but subsequently developed small clots in contact with glass. These subsequently increased in size. The time of commencement of clotting was 4′ 30″, of completion of that process 7′ 0″, the times being in each case taken from the moment of shedding. The left ventricle was empty.

Exp. 3. Rabbit died under ether. Heart immediately excised and placed in oil at 65°. During the next seventeen minutes the temperature of the oil varied from 60–67°. It was then kept at 66° for six minutes. Examination of the right ventricle showed clots on the chordae tendinae and a considerable amount of fluid blood in which four small clots formed after it was shed on to glass. The residual fluid was not precipitated by either one-fifth saturation with ammonium sulphate or by half saturation with NaCl. The blood from the left ventricle behaved in a similar manner.

Exp. 4. Cat. The conditions were the same as in Exp. 2, except that the heart had stopped beating for eleven minutes before excision. After removal from the bath all blood was found to be fluid, except for some small clots in the left auricle. The fluid blood failed to clot.

Exp. 5. Cat. Heart excised 19 minutes after death from A.C.E. No heat applied. All blood fluid but subsequently completely clotted on glass. The coagulation times of blood from the carotid before death were: commence-

ment 7′ 15″ and completion 7′ 55″. Blood from the excised heart showed commencement of clotting 5′ 10″ and completion 8′ 0″.

A comparison of Exps. 4 and 5 shows that blood shed from a dead heart at room temperature clots but that the fluid taken from the cavities of a dead heart which has been heated from 56–63° behaves like blood which has been heated to a like degree either in a test tube or in a vein exposed to the air, *i.e.* the fibrinogen is destroyed. Blood taken from the cavities of a moribund heart (as in Exp. 3) which has been heated above the coagulation temperature of fibrinogen has the bulk of its fibrinogen destroyed. When however the freshly excised and still beating heart is immersed in a hot bath and is heated for 15–20 minutes to 56–60° fibrinogen remains in the blood contained in the ventricles. Such blood clots spontaneously when shed on to glass and is precipitated by one-fifth saturation with ammonium sulphate and by half saturation with sodium chloride (Exps. 1 and 2). Evidence is thus forthcoming that fibrinogen exists in blood, when unexposed to air or foreign surfaces, after heating above the coagulation temperature of the fibrinogens of the laboratory.

The next experiment illustrates the effect of sodium citrate on intracardiac blood heated to 56·5°.

Exp. 6. Cat. 3 kilo. A.C.E. pithed. Artificial respiration. 10 cc. of a 20 % solution of sodium citrate was injected into the jugular. The animal exhibited pronounced opisthotonus and its heart stopped. Blood withdrawn by aspiration from the right ventricle failed to clot, but clotted on re-calcification. The heart was excised and heated in a normal saline bath for 8½ minutes to 56–56·5°. On opening the ventricles the blood was found to be fluid. It did not clot on recalcification.

This experiment indicates that the intravascular injection of sodium citrate alters the condition of the fibrinogen moiety of the fibrinogen-prothrombin complex of the blood or of a protective colloid associated with fibrinogen in circulating blood.

A THEORY OF THE FLUIDITY AND CLOTTING OF THE BLOOD.

In introducing this theory a preliminary paragraph is perhaps not inopportune. Any hypothesis is merely a conceptual device for the correlation of experience and can lay no claim to finality. It is in this sense that the theory to follow is suggested. Hypotheses exhibit signs of senile decay when, to maintain them, it is necessary to postulate, as knowledge progresses, an increasing number of substances not actually observed or isolated. Such indications are evident in the current thrombin theories of coagulation.

The essential characteristics of the fibrinogen of the laboratory are:

(1) It coagulates by heating to 56–57°.

(2) It is clotted by moderate amounts of thrombin.

(3) It is precipitated by one-fifth saturation with ammonium sulphate [McLean, 1920] and by half saturation with sodium chloride.

None of these features is exhibited by intravascular blood, provided its surface conditions have not been disturbed.

The disparity of this conclusion with the classic experiment of Fredericq on heating the "living test tube" to 56° becomes intelligible on consulting Fredericq's original memoir; for he found, almost invariably, a clot adjacent to the junction of the plasma and settled out corpuscles. Fibrin ferment (thrombin) was also present, although absent from the circulating blood of the horse. The colloids of this plasma had therefore progressed towards clotting.

The differences of the fibrinogen of the laboratory from that of undisturbed intravascular blood may be explicable in one of two ways:

(*a*) That fibrinogen does not exist in circulating blood, but that a precursor of it is present:

(*b*) That the fibrinogen in circulating blood is associated with a protective colloid, which renders it resistant to changes by the agencies named.

The following facts indicate that the latter interpretation is the more probable.

1. Fibrinogen is closely associated with prothrombin. On heating it can be resolved into two proteins exhibiting different coagulation temperatures [Huiskamp 1905; Iscovesco, 1906].

2. The protection against precipitation by chloroform resulting from dissolving laboratory fibrinogen in the protein residues of plasma [Nolf, 1921].

3. Both fibrinogen and prothrombin can be obtained from plasma after dilution with excess of distilled water, yet whole blood, even after contact with cut tissue, when so diluted and evaporated down to its original volume does not spontaneously clot [Pickering and Hewitt, 1921]. Lack of activation of prothrombin due to absence of "thrombokinase" cannot be pleaded as an explanation of these results, because the blood employed had been in contact with cut tissues, exhibited complete haemolysis following the dilution with distilled water and coagulated normally on glass when undiluted. The inhibition of clotting is however intelligible on the view that colloidal material is associated with the fibrinogen-prothrombin complex in circulating blood and the action of excess of distilled water may be regarded as stabilising such material.

It is suggested that the essential factors in the maintenance of the fluidity of circulating blood, in the clotting of shed blood and in the formation of intravascular clots are (1) a fibrinogen-prothrombin complex, (2) a protective colloid united to the fibrinogen-prothrombin complex in circulating blood and (3) salts of calcium.

No series of antibodies is postulated, as in the thrombin theories, but when the surface conditions of the blood are disturbed, either by shedding or by the introduction of disturbants *in vivo*, disunion of the protective colloid and the fibrinogen-prothrombin complex occurs. The inception of these changes is marked when blood slowly clots in oil by the appearance of a reversible gel [Pickering and Hewitt, 1921] and similar reversible material was found by

Pickering [1923] in the filaments formed immediately after bleeding in films of human blood exposed to moist air. From a comparison of the times of appearance of the reversible gels with the pre-clot changes in shed blood observed by Vines [1921, 1] and by Novy and De Kruif [1917], it would appear that the disunion of the protective colloid and the fibrinogen-prothrombin complex corresponds in time with the changes in the content of ionised calcium in shed blood, observed by the former worker, and with the development of toxicity recorded by the latter.

The next stage in the coagulation of the blood is the activation of prothrombin, yielding a *post-mortem* product thrombin. This may be effected by calcium ions [Howell, 1914, 1], or in blood in contact with damaged tissues by calcium ions and the hydrophile material called thrombokinase. On this view, both thrombin and thrombokinase would be regarded as accelerators of coagulation, rather than as initiators of that process. The prime factor in fluidity and clotting would thus be the surface conditions of the protective colloid, as when these remain undisturbed or if they are artificially stabilised then fluidity is preserved.

Working with fibrinogen purified by adsorption by barium sulphate and with prothrombin prepared by a modification of Mellanby's method, Dale and Walpole [1916] found that thrombokinase was necessary for the clotting of fibrinogen. Gratia [1918] found that calcium chloride, dissolved in distilled water, causes the lysis of platelets and it is generally recognised that disintegrated platelets yield thrombokinase. It is also generally accepted that prothrombin exists in circulating blood. Löwit [1892] found that the massive intravascular injection of calcium chloride fails to induce thrombosis and Pickering and Hewitt [1922, 2] obtained the same result with lethal intravenous injections of large amounts of calcium chloride, dissolved in distilled water, even after the intravascular injection of massive doses of thrombin into cats, deprived of hepatic activity. It would thus appear, accepting Dale and Walpole's results, that thrombokinase activates the prothrombin of the laboratory, but may not always invoke clotting under the conditions of circulating blood. This is intelligible as an example of protection *in vivo*, against the disturbant properties of the thrombokinase.

In the development of coagulation prothrombin is converted into thrombin and the union of fibrinogen and prothrombin is broken up. In this sense, the process of clotting is the lysis of a colloidal complex. Evidence is not wanting as to the similarity of protection against the precipitation or the coagulation of blood and protection against haemolysis. Numerous observers have shown that serum protects against haemolysis by several agencies, and Nolf [1921] has shown that the proteins of serum protect fibrinogen against precipitation by chloroform. The addition of egg-albumin to shed blood has long been known to delay clotting, and egg-albumin protects against haemolysis by eosin in the presence of sunlight [Schmidt and Norman, 1920], and also against haemolysis by saponin and by a heterologous serum [Pickering and Taylor, 1923].

The addition of an equal volume of 0·5 % solution of sucrose to blood inhibits coagulation. Eisler [1909] found that sucrose protects against specific haemolysis and observed that the addition of moderate amounts of electrolytes restores haemolytic action. Isotonic sucrose also inhibits the haemolytic action of saponin and sapotoxin [Furuhata, 1918]. "Peptone" inhibits the clotting of blood, and Schmidt and Norman [1920] have shown that "peptone" protects against haemolysis by eosin, in the presence of sunlight. Coagulability is restored to "peptonised" blood by the addition of moderate amounts of sodium chloride, by neutralisation with a weak acid and by the passage of carbon dioxide.

We have seen that the addition of moderate amounts of electrolytes restores haemolysis after protection by sucrose, and Pickering and Taylor [1923] have found that either acid or neutral egg-albumin affords less protection against haemolysis by saponin than does alkaline egg-albumin.

If the view is accepted that the problems of the fluidity and clotting of blood are comparable to those of the protection against, and the inception of, haemolysis, then they may ultimately be resolved into questions of the interfacial permeabilities of the fibrinogen-prothrombin complex of the plasma.

There remains for consideration a number of generally recognised phenomena in the fluidity and coagulation of blood, some of which have been neglected by the supporters of the thrombin theories, but all of which can be interpreted on the hypothesis just enunciated.

(1) *The fluidity of circulating blood.* It may be assumed that the surface conditions of the vascular wall are such as not to disturb those of the protective colloid of the plasma.

(2) *The condition of the plasma in the excised jugular of the horse.* Here partial clotting occurs with the formation of thrombin [Fredericq, 1878], but the bulk of the plasma remains fluid. This the thrombin fails to clot, in like manner to the failure of thrombin to clot circulating blood. This may be attributed to absence of disturbance of the protective colloid in those portions of the plasma in contact with the undamaged vascular wall.

(3) *The slowing of clotting on oiled and paraffined surfaces.* This is intelligible on the view that oil or paraffin only slowly causes surface change in the protective colloid of the plasma.

(4) *The fluidity of bird's blood* in vitro. This can be explained by regarding the protective colloid in bird's blood as more stable than that in mammalian blood. The observation of Rettger [1909] that bird's blood *in vitro* is clotted by a feather is explicable by disturbance of surface conditions by that agency. Coagulation by the rapid addition of tissue extract (thrombokinase) may be regarded as acting in a like manner.

(5) *The effect of re-precipitating and washing fibrinogens.* Howell [1916] found that the fibrinogens prepared from the oxalated plasmas of the bird and of the terrapin, by a single precipitation with sodium chloride, frequently

failed to clot on the addition of thrombin; but that the fibrinogens prepared by two or more precipitations were clotted by thrombin, provided the precipitates were sufficiently washed. This is intelligible on the view that the repetition of precipitation and washing disassociates the fibrinogen from the protective colloid.

(6) *The coagulant action of a thread.* The following type of experiment was devised to elucidate this.

Exp. 7. Cat. A.C.E. pithed. Artificial respiration for 20′ before operating on the heart. Three cotton threads were introduced into the right and left ventricles, by passing threaded needles through the heart. The coagulation times of carotid blood, shed immediately before the introduction of the threads, were commencement of clotting 5′ 50″ and completion of that process 7′ 0″. A mass ligature was placed round the heart, which was excised while beating. Examination showed large clots surrounding the threads and some fluid blood. The latter on shedding on glass commenced to clot in 1′ 0″ and was completely clotted in 1′ 20″.

The preceding experiment indicates a sharp distinction between the residual fluid blood after the formation of mechanically produced thrombi and the blood remaining fluid after the formation of thrombi by the slow intravascular injection of tissue extract. In the former case the residual blood is hypercoagulable, in the latter it exhibits inhibition of clotting, the negative phase of Wooldridge. The hypercoagulable blood when shed closely resembles, in the speed of its clotting, blood shed after the intravascular injection of thrombin, while blood in the negative phase behaves like blood to which an anticoagulant has been added.

The production of a thrombus by a thread is intelligible as an example of the processes whereby a thread hastens the coagulation of shed blood. The electropositive ions in the fluid adjacent to the thread (the latter when wetted being electronegative) may well be regarded as disturbants in the first instance of the protective colloid and later of the fibrinogen-prothrombin complex. The fact that a considerable amount of coagulable blood was found associated with the thrombus indicates that any thrombin formed had no coagulant action on that portion of the blood whose surface conditions had not been disturbed. This may be attributed to the presence of undisturbed protective colloid, preventing the action of thrombin on the fibrinogen of the blood. In like manner may be explained the fact that the localised clot formed when an artery is ligatured does not lead to general thrombosis.

(7) *The action of organic intravascular coagulants.* Tissue extracts, some snake venoms and certain synthesised substances [Pickering, 1896], when rapidly injected into the circulation, produce intravascular clotting. This is intelligible on the view that the hydrophile qualities of these substances alter the surface conditions of the colloidal complexes of the plasma. Change in the distribution of electrolytes may also play a part.

(8) *The addition of a small quantity of water.* This hastens coagulation

in vitro and well may be a disturbant of any protective colloid present. It is noteworthy that Howell [1912] found that the addition of a small quantity of water to the shed blood of the terrapin induced clotting, but that addition of isotonic saline had no such effect.

(9) *The action of neutral salts on shed blood.* Relatively stable unions of these salts with either the protective colloid or the fibrinogen-prothrombin complex or both would account for the inhibition of clotting obtained. This view is concordant with the observation of Wooldridge [1893] that thrombin is absent from salted plasmas.

(10) *The action of oxalates and citrates*[1]. This is probably dual in nature, a union of the salts with the protective colloid and/or with the fibrinogen-prothrombin complex, and also the removal of calcium, or, in the case of citrates, change in the ionisation of the calcium present. The former may stabilise the protective colloid, the latter may modify the subsequent activation of prothrombin. In the case of citrated blood or plasma changes in dispersion may also play a part.

Exp. 6 shows that the intravascular administration of sodium citrate disturbs the colloidal complexes of the plasma, for the fibrinogen of intraventricular citrated blood coagulates at the same temperature as does the fibrinogen of the laboratory.

The action of citrates *in vitro* is progressive. Freshly citrated blood is coagulated by addition of a non-retracted clot and by tissue extracts, but blood 18 to 23 hours after citration is not clotted by these agencies [Folley, 1918]. These facts are intelligible as due to the gradual adsorption of citrate by the protective colloid of the plasma, yielding, after prolonged action, a more stable protective complex than is at first formed. The fact that blood citrated for 18 to 23 hours can be clotted by the addition of small amounts of sodium chloride (Folley) is significant in connection with the clotting of "peptone" plasma by sodium chloride and in relation to the antagonistic action of certain concentrations of electrolytes to protection against haemolysis.

(11) *The inhibition of clotting by "peptone," nucleic acids and hirudin.* The anticoagulant action of "peptone" and of nucleic acids has been explained as due to excessive secretion, by the liver, of antithrombin or of antithrombosine [Nolf, 1910]. But Pickering and Hewitt [1922, 1] have shown that inhibition of clotting occurs after the rapid intravascular injection of "peptone" into cats deprived of hepatic activity, and unpublished work by the same authors indicates that nucleic acids (freed from proteoses) exhibit a like effect under like conditions. It has also been shown that the addition of "peptone" to blood or plasma *in vitro* inhibits clotting, provided adequate precautions have been taken to preserve the surface conditions of the latter fluids [Pickering and Hewitt, 1922, 1].

On the view of coagulation enunciated, the anticoagulant action of "peptone" and of nucleic acids may be ascribed to the union of these sub-

[1] Work is in progress in this laboratory on citrates and fluorides.

stances with the protective colloid of the plasma, yielding a more stable protective complex than that existing in circulating blood. Several of the generally recognised reactions of peptonised blood point to this conclusion. It is clotted by dilution with water, by the passage of carbon dioxide and by filtration through a clay cell. Each of these agencies, as Wooldridge [1893] pointed out, tends to dissolution. The disruption of a relatively stable union of a protective colloid and "peptone," under the conditions of shed blood, may be expected to cause clotting. "Peptonised" blood ultimately clots, when exposed to the air in glass vessels. This is intelligible as the gradual resolution, under the stress of disturbed surface conditions, of the union of the "peptone" with the protective colloid of the plasma. The addition of tissue extract rapidly coagulates "peptonised" blood. This may be attributed to lysis by the hydrophile material of the tissue detritus. On the other hand, thrombin fails to clot "peptonised" blood [Wooldridge, 1893]. On the view advocated, the difference between "peptonised" and normal shed blood is that in the former the protective colloid is stabilised by union with "peptone," in the latter it has undergone surface change. Thrombin only acts as a coagulant after surface change has disunited the union of protective colloid and the fibrinogen-prothrombin complex of the plasma. In "peptonised" blood this union is preserved and thrombin has consequently no action.

Hirudin exhibits its anticoagulant action after injection *in vivo* and admixture *in vitro* and its union with the protective colloid of the plasma is more stable and less toxic than are the unions of plasma with "peptone" or nucleic acids. Its action and its neutralisation by tissue extracts are explicable on the views stated. At a later stage in coagulation, hirudin also apparently combines with thrombin [Vines, 1921, 2]. The fact that "peptone" has no anticoagulant action on blood shed in the absence of precautions to preserve its surface conditions points to the union of the "peptone" with the unchanged protective colloid of the plasma. Hirudin, however, may unite with the colloids of the plasma, after disturbance of their surface conditions. Nucleic acids occupy an intermediate position, for they exhibit anticoagulant action after intravascular injection, after addition to blood shed on paraffined surfaces and also when blood is shed on glass. The degree of anticoagulant action is greatest when nucleic acids are injected *in vivo*, least when added to blood shed on glass, the action on blood shed on paraffined surfaces occupying an intermediate position.

(12) *The negative phase of coagulation.* No satisfactory explanation is afforded by the thrombin theories. A negative phase in the coagulation of bird's blood *in vitro* has been produced by Pickering and Hewitt [1921] by the slow addition of tissue extract, drop by drop, to that fluid. In the paper recording this fact attention was directed to the negative phase or tolerance exhibited in the precipitation of colloids by the slow addition of electrolytes, in precipitation of gelatin by the slow addition of alcohol, in the fractional neutralisation of certain toxins by antibodies (known as the Dansyz [1902]

reaction), and in the fractional neutralisation of the toxic qualities of arsenious acid by ferric hydroxide. It was submitted that these phenomena are of one class and are explicable in the same manner as physico-chemical processes.

On the view that a protective colloid is associated with the fibrinogen-prothrombin complex of the plasma, the *rapid* addition of tissue extract *in vivo* or *in vitro*, possibly by creating inequality of electric charge, tends to dissolution of the protective colloid and to coagulation; the *slow* addition of the same substance tends to uniformity of charge and to the formation of unions with the protective colloid, which unions (of protective colloid and tissue extract) exhibit physical resistance to further disturbance by the tissue extract.

In this connection, the reversed or negative action of "peptone" on the coagulability of blood demands notice. "Peptone" when introduced into the circulation slowly, drop by drop, not only does not delay clotting but produces partial or complete immunity against the anticoagulant action of subsequent injections of "peptone." This occurs both in intact animals and in those deprived of hepatic activity. This immunity reaction is intelligible on the view that the slow introduction of the "peptone" promotes unions with protective colloid of the plasma, which unions exhibit physical resistance against further unions with "peptone."

(13) *The action of silica sols.* These afford a marked contrast to the action of tissue extracts. Gye and Purdy [1922] found that lethal intravenous injections produced extensive thrombosis, that fractional injections induced hypercoagulability and that their addition to shed blood did not alter the speed of clotting. If we accept the conclusion of these observers that the lethal injections damaged the vascular wall, then the thrombosis is explicable. For damage to the vascular wall alters those surface conditions, which are essential to the preservation of the stability of the protective colloid of the plasma. The absence of a negative phase and the lack of coagulant action *in vitro* indicate that sols of silica neither unite with nor directly disintegrate either the protective colloid or the fibrinogen-prothrombin complex.

(14) *The influence of temperature.* That cooling retards clotting and heating hastens that process are generally accepted, but no unanimity exists among observers as to the variations in the speed of spontaneous clotting arising from increasing temperatures. This may be accounted for by the wide divergences in the methods adopted to determine coagulation time, and by the varying degrees of contact of the blood with damaged tissues. In the experiments to be described attempts were made to eliminate or minimise this.

Exp. 8. Blood was shed from the carotid of a pithed cat through an evenly paraffined cannula into wide mouthed glass vessels each of the same calibre. The vessels were submerged above the level of the blood in them in water-baths, raised to the desired temperatures. Evaporation was minimised by covering the vessels with moist filter papers. As it is known that hypercoagulability may result from successive haemorrhages, observations at any one temperature were repeated at different stages of the bleeding.

The graph (Fig. 1) shows the speeds of clotting corresponding to temperatures ranging from 33–57°. The points on the upper line of the graph represent the times of completion of coagulation, those on the lower line show the times of commencement of that process.

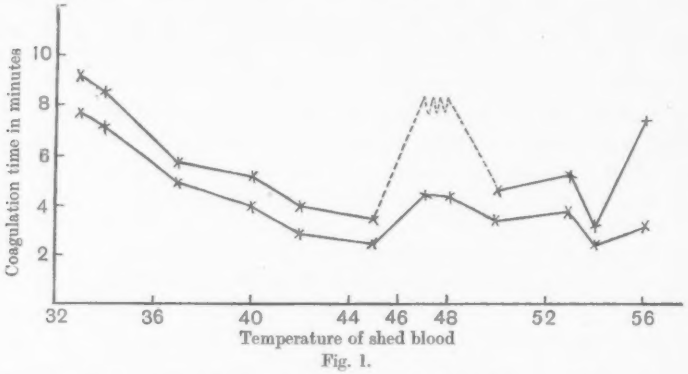

Fig. 1.

Exp. 9. Human blood was obtained by bleeding from serial punctures in the fingers, after cleaning with chloroform and ether and coating the skin with paraffin wax. The paraffined surface was cleansed after each bleeding. The points in the graph (Fig. 2) have the same significance as in Fig. 1. In this experiment contact with damaged tissue is not eliminated, but contact with clotted blood and foreign material is minimised.

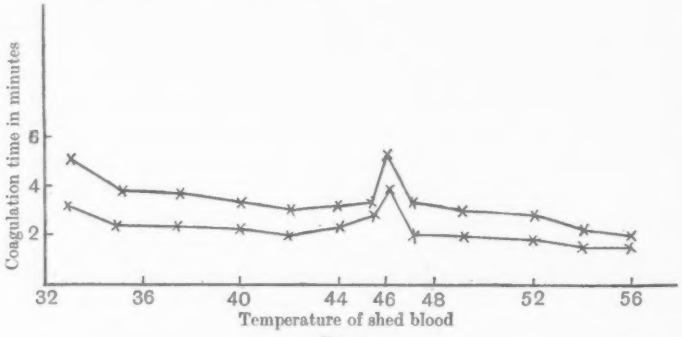

Fig. 2.

Irregular results were obtained in other experiments in which the same puncture was repeatedly used, when bleeding was not on paraffined surfaces and when pressure was exerted to squeeze out the blood.

The following is indicated by Fig. 1: (*a*) In the absence of contact with cut tissues and at temperatures ranging from 33 to 42°, the speed of spontaneous coagulation of the blood of the cat shed on glass increases regularly

with rises of temperature. (*b*) From 42 to 45° increase of temperature increases the speed of clotting, but the acceleration is not so marked as with increases at lower ranges of temperature. (*c*) At 47° marked slowing occurs in the commencement of coagulation and the end point of clotting is not well marked. The type of clot underwent profound change on heating to 47–48°. At lower and higher temperatures (below 56°) clotting was completed by the formation of a continuous gel, so that the vessel could be inverted without spilling blood. At 47–48° a succession of small clots was formed and the phase of a continuous gel was absent. The small clots contracted and progressive lysis of these clots was observed. It is significant that these changes in coagulability and the appearance of lysis of clots occur at a temperature which corresponds with that at which death and the onset of heat rigor is manifested in mammalian muscle [Brodie and Richardson, 1899]. In this connection, the views of Nolf [1909] on the supposed metabolic importance of the lysis of clots and the assumed secretion of antifibrinolysin by the liver may be recalled.

The second graph (Fig. 2) illustrates the retardation of the speed of clotting at 46° obtained with human blood which had passed through punctured tissue. With this blood the end point of coagulation was marked by the formation of a continuous gel.

These results are concordant with the view that physical or chemical changes or both occur either in the protective colloid or in the fibrinogen-prothrombin complex at the time of the death of the blood. Whether the lysis of the complexes of plasma is due to death by heat future experiment must determine. From a number of preliminary experiments on heating pithed animals to 47–49° it is evident that coagulability is not only modified but that intravascular clots may be produced. It is proposed to return to this subject later.

Summary.

(1) Blood in the freshly excised heart, with its cavities unexposed to air, can be heated for 15 to 20 minutes to 56–60° without the destruction of fibrinogen. Such blood coagulates when shed, is precipitated by one-fifth saturation with ammonium sulphate and by half saturation with sodium chloride.

(2) Blood in a dead heart when heated under like conditions has its fibrinogen destroyed. Blood in a moribund heart has its fibrinogen partially destroyed.

(3) If intraventricular blood is citrated and recalcified *in vivo*, its fibrinogen is completely destroyed on heating, without exposure to the air, to 56–56·5° for 8½ minutes.

(4) A theory of the fluidity and the coagulation of the blood is enunciated. The observations recorded and the generally recognised phenomena of fluidity and clotting are correlated by this hypothesis.

(5) Attention is directed to the similarity of phenomena in protection against the spontaneous coagulation of blood and those of protection against haemolysis.

(6) The fluid blood adjacent to mechanically produced thrombi is hypercoagulable. Attention is directed to the contrast in the condition of this blood with that of the fluid blood associated with the thrombi formed in the negative phase of clotting. Reasons are given why the thrombus produced by ligaturing a living artery does not produce general thrombosis.

(7) Observations are recorded on the influence of heat on the coagulability of the shed blood of the cat, after precautions had been taken to prevent contamination with tissue detritus. The influence of heat on the coagulability of human blood after contact with the surfaces of a puncture, is also described. Both these bloods exhibit marked changes in coagulability on heating to 46–48°, *i.e.* at the temperature of death and the onset of heat rigor in muscle.

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XCII. AN IODOMETRIC METHOD FOR THE ESTIMATION OF NITROGEN IN OSAZONES.

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(*Received August 30th, 1923.*)

It is well known that those compounds containing nitrogen atoms directly linked together do not respond quantitatively to the Kjeldahl process, and for the estimation of nitrogen in such cases resort must be had to Dumas' method. Now although this method leaves nothing to be desired on the score of accuracy, it possesses certain disadvantages. Thus, apart from the question of time and attention required, it not infrequently happens that the quantity of material at disposal does not amount to more than a few decigrams. Among those compounds to which these remarks apply may be mentioned phenylhydrazine and its derivatives, and the method to be described was devised more especially for estimating nitrogen in osazones.

Grimaldi [1903] has suggested a method for the estimation of hydroxylamine in oximes, and phenylhydrazine in hydrazones and osazones by heating these compounds in a flask having a long narrow neck with a known volume of standard hydrochloric acid in excess in a boiling water-bath for one hour. The aldehyde or the ketone is regenerated and the liberated hydroxylamine or phenylhydrazine forms the hydrochloride. The excess of the uncombined acid is then titrated with $N/100$ alkali. From the amount of acid neutralised the hydroxylamine or phenylhydrazine may be calculated. This method appeared to be a convenient one for the estimation of nitrogen in osazones of sugars. The original communication not being available for consultation I am without details of the author's procedure. Some preliminary experiments were therefore carried out to determine the concentration of acid necessary for decomposing the osazones. It was found that $N/10$–$N/100$ hydrochloric acid did not act on glucosazone under these conditions, but N hydrochloric acid brought about the expected decomposition.

The method entailed the titration of N acid with $N/100$ alkali. It was therefore useless for delicate work and had to be abandoned.

The reaction still seemed interesting and it was investigated in another way. Instead of titrating the excess of the acid after decomposing the osazone under the above conditions it appeared possible to estimate the phenylhydrazine formed during the reaction by the iodometric method described in the

previous paper by Ling and Nanji [1921]. This method of estimating phenylhydrazine depends on the fact that when a solution of phenylhydrazine is added to an excess of a standard solution of iodine it is decomposed according to the equation:

$$C_6H_5 . NH . NH_2 + 2I_2 = C_6H_5I + N_2 + 3HI.$$

The reaction is an extremely sensitive one and has proved very useful in studying the decomposition of osazones with hydrochloric acid. The method employed was as follows.

A known weight of the osazone was heated with a known volume of *N* hydrochloric acid in a boiling water-bath for one hour. The reaction mixture was then neutralised with an equivalent amount of standard alkali, acidified with a drop of dilute acetic acid, and then made alkaline with an excess of pure sodium bicarbonate solution. The mixture was then washed into an excess of a known volume of standard iodine solution and the excess of iodine titrated with sodium thiosulphate, the difference being equivalent to the phenylhydrazine formed in the reaction mixture.

It was found by studying the above reaction between osazones and hydrochloric acid by this means that the decomposition did not proceed quantitatively, but it proceeded to an extent which was determined by the relative amounts of nitrogen present in the sample and the volume of the hydrochloric acid used. When the amount of hydrochloric acid was kept constant and the amount of nitrogen increased the percentage of the osazone decomposed decreased steadily. As a result of a large number of titrations it was observed that so long as the ratio of the nitrogen to the hydrochloric acid used was fixed, the percentage of the osazone decomposed was constant. Accordingly a curve was obtained by keeping the amount of hydrochloric acid constant and varying the amount of nitrogen present. When this curve is applied to different sugar osazones very concordant results are obtained. This has been made the basis of an empirical method for the estimation of nitrogen in sugar osazones, and it gives trustworthy and accurate results as will be seen by the following experiments, so long as the estimations are carried out under definite conditions.

The method is as follows:

A convenient weight of the osazone which may vary from 5–60 mg. is accurately weighed out in a Freudrich flask. To it is then added 10 cc. of *N* hydrochloric acid, and the mixture heated in a boiling water-bath for one hour. The reaction mixture is washed into a beaker and to it is added 10 cc. of *N* sodium hydroxide. It is then acidified with a drop of dilute acetic acid and the solution made alkaline with an excess of pure sodium bicarbonate solution. The alkaline solution is then washed into an Erlenmeyer flask concontaining an excess of a known volume of standard *N*/50 iodine solution. The excess of the latter is then determined by titrating with a standard solution of sodium thiosulphate. The number of cc. of *N*/50 iodine used on being

referred to the following table corresponds to the amount of nitrogen present in the sample taken, from which the percentage could be calculated.

cc. of *N*/50 iodine used	mg. of nitrogen	cc. of *N*/50 iodine used	mg. of nitrogen	cc. of *N*/50 iodine used	mg. of nitrogen
4·7	0·782	14·0	3·371	22·0	6·757
5·0	0·839	15·0	3·628	23·0	6·980
6·0	1·029	16·0	3·885	24·0	7·203
7·0	1·219	17·0	4·142	25·0	7·426
8·0	1·409	17·3	4·220	25·7	7·582
9·0	1·599	18·0	4·428	26·0	7·679
9·3	1·656	19·0	4·723	27·0	8·002
10·0	1·958	19·1	4·752	28·0	8·325
11·0	2·389	20·0	5·972	28·6	8·518
12·0	2·820	20·3	6·378	29·0	8·754
12·2	2·906	21·0	6·534	29·5	9·049
13·0	3·114	—	—	—	—

The following are some of the results obtained with different osazones. They represent the mean values of a number of estimations with quantities of the osazone employed varying from 5–60 mg.

Glucosazone	N	Found	15·52 %	Calculated	15·64 %
Maltosazone	N	,,	10·56	,,	10·76
Isomaltosazone	N	,,	10·65	,,	10·76
α-Glucosido-isomaltosazone	N	,,	8·14	,,	8·21

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XCIII. STUDIES ON CARBOHYDRATE METABOLISM.

III. ON THE FORMATION OF AN ANTI-DIABETIC HORMONE BY THE ACTION OF A BACILLUS. (PRELIMINARY COMMUNICATION.)

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(*Received August 28th, 1923.*)

Working with samples of commercial yeast, it was found [Winter and Smith, 1923] that an extract could be prepared which had an effect similar to insulin when tested on normal animals and diabetic persons. Later it was found [Hutchinson, Smith and Winter, 1923] that extracts from different samples of commercial yeast differed widely in their activity. It was improbable that these differences were due to variations in the nutritional state of the yeast, and it seemed possible that the different commercial samples of yeast might contain quantities of organisms, which are responsible for the whole of the insulin-like activity. It has been found that certain yeasts in pure culture give appreciable quantities of an anti-diabetic hormone, while others are quite inactive in this respect. The possibility was not excluded, however, that there might be in commercial yeast some micro-organism, which was not a yeast, but which was capable of forming an insulin-like substance.

The following is an account of some work directed to test this possibility. The anti-diabetic hormone will be referred to as glucokinin, though it has been pointed out [Hutchinson, Smith and Winter, 1923] that the substance so named and described by Collip may be different from that which we propose to discuss.

A portion of a sample of yeast was extracted with alcohol and the activity of the extract tested on rabbits. The blood sugar determinations were made by Bang's old method. The effect on the blood sugar was to cause a moderate fall, as will be seen from the following experiment.

Rabbit weight 1·8 kg.

Time		Blood sugar %
10·00		·14
10·10	Injected ·06 mg./g.	
12·00		·10
3·00		·12
5·30		·14

A portion of the same yeast was plated out on nutrient agar and a pure culture of a bacillus obtained. This was sown into 100 cc. peptone water containing 1 % glucose, and incubated at 37°. Rapid growth took place, and after two days alcohol was added to make the mixture 75 %. The fluid was placed in the ice-chest over-night, filtered, and concentrated to 20 cc. It was then made up to 80 % with alcohol and allowed to stand in the ice-chest for 12 hours. The supernatant fluid was then made up to 95 % with alcohol, when an appreciable precipitate was formed. Precipitation was complete in 24 hours at − 7°. The precipitate was washed with absolute alcohol, and dry ether, and dried. This powder was tested on rabbits, the standard injection being given (·06 mg./g. body weight). A marked fall in the blood sugar occurred. It appeared probable therefore that an appreciable amount of glucokinin had been formed by the action of the bacillus. Control experiments were performed in which the sterile medium was extracted with alcohol, and the resultant powder injected into a rabbit. No fall in the blood sugar occurred, though the yield of extract was similar in quantity.

Rabbit weight 1·9 kg.

Time	Blood sugar %
10·15	·12
10·30	Injected (bacillus extract)
11·45	·09
1·00	·08
3·30	·12

Rabbit weight 2 kg.

Time	Blood sugar %
10·00	·10
10·15	Injected (control)
11·30	·10
12·50	·11
2·30	·10

In the previous experiments in which the bacillus was grown in the glucose peptone water the reaction at the end of the experiment was found to be markedly acid. Since it is well known that phosphates are of importance in the metabolism of carbohydrate, the addition of phosphate was employed, both for this reason, and for the purpose of controlling the reaction of the fluid. The bacillus was sown in the following media.

I. 200 cc. peptone water containing 1 % glucose and 1 % Na_2HPO_4.

II. The same as above, but with NaH_2PO_4 in place of the di-sodium salt.

Growth was allowed to continue for four days when the extracts were made as before. The activity was greater in the slightly alkaline medium, the following being results obtained from tests on rabbits.

Rabbit weight 2 kg. Na_2HPO_4.

Time	Blood sugar %
11.00	·10
11.15	Injected
12.20	·08
3.00	·06
4.45	·05
6.00	·04
6.10	Convulsed
6.20	Glucose injected
6.45	Animal eating
9.00	·11

Rabbit weight 1·7 kg. NaH_2PO_4.

Time	Blood sugar %
10.00	·09
10.15	Injected
11.30	·07
1.00	·09
3.00	·10

In another experiment in which tri-sodium phosphate was used, no growth occurred. In the above experiment in order to make the conditions com-

parable the reaction was adjusted in each case to p_H 5 before concentration *in vacuo*. Though it is uncertain whether the substance produced by the bacillus has similar properties to insulin, the known stability of insulin to acid suggested that concentration should preferably be carried out in acid solution. We have since found, however, that concentration can be carried out in a slightly alkaline medium. Some further experiments have been performed with a view to comparing the yield of active principle obtained under different conditions. The strength of glucose in the peptone water was varied from 1 to 2 %. No appreciable variation in the activity was found. In another experiment the bacillus was sown in peptone water without glucose in the presence of 1 % Na_2HPO_4. Growth was allowed to continue for four days. Moderate activity resulted. The following shows the results of experiments with batches grown with and without glucose.

With glucose Rabbit weight 2 kg.		Without glucose Rabbit weight 2·5 kg.	
Time	Blood sugar %	Time	Blood sugar %
11.00	·10	10.00	·11
11.15	Injected	10.15	Injected
12.20	·08	11.30	·08
2·30	·06	12.30	·07
4.30	·05	2.00	·09
6.00	·04	3.30	·10
6.10	Convulsions		
6.20	Glucose		
6.35	Animal eating		
8.00	·11		

To determine the effect of aeration on the production of glucokinin, the bacillus was sown in peptone water containing 1 % glucose and di-sodium phosphate. The mixture was incubated at 37°, a stream of sterile air being drawn through the mixture for two days. Abundant growth took place. At the end of this period the action was stopped, and the usual alcoholic extract made. On testing this however on rabbits it was found that the activity was much diminished.

In view of the considerable quantities of alcohol necessary, experiments were carried out in which the medium in which the bacillus had grown was concentrated *in vacuo* without preliminary treatment with alcohol. 250 cc. of medium were concentrated to 25 cc. and made up to 80 % with alcohol. The precipitate formed was very large. When this had settled the supernatant fluid was made up to 95 % alcohol as before. The resulting precipitate contained only a small amount of active principle. Presumably the first precipitation in a large quantity of alcohol tends to remove interfering substances without causing appreciable loss of glucokinin, but when these substances are precipitated in a small quantity of 80 % alcohol the glucokinin is largely carried down with them. Further experiments are being made in order to remove these substances in the first stage by another method without the use of alcohol.

Discussion.

That the convulsions caused by the injection of an extract of the medium in which the bacillus had been grown were not due to a toxic effect was shown by the fact that the animals invariably recovered as a result of injection of glucose. The effect therefore would seem to be similar to that produced by insulin or an extract of yeast with consequent hypoglycaemic convulsions. The active principle obtained by the action of the bacillus would appear to be similar to that obtained from yeast since in both cases the same delayed action is noticed. Convulsions usually occurred six hours after injection in the case of an active extract. Only one injection was necessary to cause convulsions in the case of such an extract.

The order of the yield obtained is as follows. 200 cc. of medium in which growth had taken place for four days yielded 0·6 g. of powder of which 120 mg. caused convulsions in a rabbit of 2 kilos.

We have not yet identified the organism used in these experiments. It is a short coli-form bacillus, producing acid and gas from glucose or lactose in 12 hours. It does not liquefy gelatin. Similar experiments carried out with a strain of *B. coli* (for this we are indebted to Miss M. Stephenson) showed that no active principle was formed. Experiments are in progress to determine whether other bacilli of this group can form glucokinin.

Summary.

1. An insulin-like substance is formed by the action of a coli-form bacillus.

2. Injection of the extract into rabbits causes hypoglycaemic convulsions which are relieved by glucose.

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XCIV. THE EVALUATION OF THE PURITY OF VARIOUS ORGANIC PRODUCTS BY THE DICHROMATE METHOD.

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(*Received August 21st, 1923.*)

The writer has previously described [1914] a method for the volumetric estimation of carbon, the essence of which is oxidation by a mixture of phosphoric acid and potassium dichromate and the measurement of the CO_2 produced. It was shown that where methyl groups are present the substance is not oxidised to carbon dioxide alone but to a mixture of carbon dioxide and acetic acid; for example, lactic acid, alanine and ethyl alcohol each yield one molecule of acetic acid per molecule of substance taken; that is to say each methyl group gives rise to one molecule of acetic acid. The method proved of service in estimating the amount of such substances as mannitol and glycerol or, in the case of a mixture, of the residual oxidisable material after a fermentation. It is possible with a few cc. of solution to estimate how much material remains after fermentation, and so on.

At the time of publishing this method the writer was unaware of Martin's method [1904] for the estimation of alcohol. Martin's method is now probably fairly generally employed in industry; certainly in France. It is exceedingly accurate and is applicable to very dilute solutions of alcohol. Martin distils the alcoholic solution into a mixture of equal parts of *N* potassium dichromate and sulphuric acid; the steam keeps the mixture hot and the oxidation is immediate, the alcohol being oxidised to acetic acid (1 cc. *N* dichromate = 0·0115 g. alcohol). The measurement of the reduction of standard dichromate solution is made by a solution of iron of about a fifth the strength of the dichromate, which increases the speed and accuracy with which one can work. The method is clearly much simpler than my own but is without some of its advantages; one cannot for instance apply it to the oxidation of a substance without knowing whether it is oxidised to acetic acid or to carbon dioxide or both. Provided however we know what are the products of oxidation, we could probably employ the dichromate method for estimating many substances; and its application to the estimation of impurities in non-oxidisable substances seems obvious.

In the second place, by a combination of Martin's method with my own one can obtain two constants which permit the estimation of the proportions of many substances, *i.e.* (1) the dichromate method, measuring the dichromate

reduced; (2) the dichromate method measuring the CO_2 produced and when necessary the acetic acid formed.

As special apparatus is required for the measurement of CO_2 it is much more convenient to employ method (1) where one constant suffices, as in estimating the amount of any pure substance.

In calculating the dichromate value of any aliphatic substance the rule is that the substance is completely oxidised to CO_2 except the carbon atom of a methyl group and the next adjacent carbon atom which appear as acetic acid; thus

$$CH_3 . CH(OH) . COOH + 2[O] = CH_3 . COOH + CO_2 + H_2O,$$

or for each cc. *N* lactic acid we require 4 cc. *N* dichromate.

Exp. 1. 25 cc. of a lactic acid solution = 27·4 *N*/10 were mixed with 11 cc. *N* potassium dichromate, and 25 cc. concentrated sulphuric acid added rapidly. The excess dichromate was titrated with ferrous sulphate solution. Found:

27·4 cc. *N*/10 lactic acid reduced 10·77 cc. *N* dichromate;
1 cc. *N* lactic acid reduces 3·93 ,,
Theory 4·00 ,,

The experiment was repeated using twice the concentration of dichromate. Found:

27·4 cc. *N*/10 lactic acid reduced 11·27 cc. *N* dichromate;
or 1 cc. *N* lactic acid reduces 4·11 ,,
Theory 4·00 ,,

Thus even with a wide range of oxidising agent (doubling the dichromate) the reaction follows the above equation. The acetic acid produced is clearly not oxidised further, in agreement with what the writer previously found [1914].

On the other hand, substances devoid of CH_3 groups should, according to the writer's theory, yield no acetic acid but only CO_2 and H_2O. The following were tried: malonic, tartaric and citric acids, ethylene glycol; with the following results:

Exp. 2. The weight of substance mentioned in each case was dissolved in 10 cc. of water and 22 cc. *N* dichromate added. To this mixture 25 cc. concentrated sulphuric acid were added. The excess of dichromate was titrated by ferrous sulphate solution.

Results:

Ethylene glycol	0·1274 g.	reduced 19·94	cc. *N* dichromate
		Theory 20·55	,,
Citric acid	0·0931 g.	reduced 7·87	,,
		Theory 7·98	,,
Malonic acid	0·1932 g.	reduced 14·86	,,
		Theory 14·93	,,
Tartaric acid	0·1035 g.	reduced 3·71	,,
		Theory 3·76	,,

The results are, within experimental error, in support of the theory.

The only exception to the rule seems to be succinic acid. This peculiar behaviour of succinic acid in resisting oxidation has previously been described. Under the conditions detailed above, succinic acid was not appreciably oxidised.

Exp. 3. 20 cc. *N*/20 succinic acid were mixed with 22 cc. normal potassium dichromate and to this 25 cc. concentrated sulphuric acid added. The excess of dichromate required for reduction 124·3 cc. ferrous sulphate solution; control dichromate and sulphuric without succinic acid required 126 cc.

2 cc. ferrous sulphate corresponds to 0·4 cc. dichromate and this represents an oxidation of at most 1·2 cc. of the succinic acid solution, *i.e.* 5 %. Allowing for error, we may say that from 2 to 5 % was oxidised; but the oxidation is often less than this.

By choosing the right concentration of reagents therefore it is possible to avoid the oxidation of any appreciable amount of succinic acid present. The concentration recommended is as follows:

(*a*) Water containing the unknown, 10 cc.
(*b*) *N* potassium dichromate 10
(*c*) Sulphuric acid, conc. 20
Add (*c*) rapidly to the mixture of (*a*) and (*b*).

It has been shown that with the exception of succinic acid, aliphatic substances follow the rule described by the writer. We may therefore calculate directly what the oxygen equivalent of any substance will be, and it is a simple matter to construct tables for mixtures of any two substances. The following table shows the application of this principle.

Table I. *Mixture of two alcohols.*

1 cc. *N* dichromate oxidises		1/6	mol. (in mg.)	methyl alcohol	or	0·00533 g.
,,	,,	1/4	,,	ethyl	,,	0·0155
,,	,,	1/10	,,	propyl	,,	0·0060
,,	,,	1/16	,,	butyl	,,	0·004623

To draw up the table we calculate how much dichromate corresponds to one decigram of each of the two substances. The difference between these two values we may divide up into a hundred parts; we can then read off at a glance the percentage of both constituents corresponding to the dichromate titration value obtained.

Dichromate values of alcohols.

0·1 g. methyl alcohol	reduces	18·740	cc. *N*	dichromate
0·1 g. ethyl	,,	8·695	,,	
0·1 g. propyl	,,	16·670	,,	
0·1 g. butyl	,,	21·620	,,	

The marked difference between the oxygen values of methyl, ethyl and other alcohols makes the method of useful application in the case of a mixture of two alcohols of which ethyl alcohol is one.

Polyhydric alcohols also follow the same rule:

0·1 g. ethylene glycol reduces 16·120 cc. *N* dichromate
0·1 g. glycerol ,, 15·220 ,,
0·1 g. mannitol ,, 14·285 ,,

All these figures have been verified by experiment except butyl alcohol. By a simple calculation from the weight taken and the dichromate value, the proportions in the case of a mixture of two alcohols such as methyl and ethyl alcohol, or ethyl and propyl alcohol can be ascertained.

Table II. *Mixtures of any two acids.*

The acid mixture should be approximately decinormal. The mixed acids are first titrated or the titre estimated by neutralising with lime and estimating the calcium. The oxygen value of 1 cc. of a normal solution must be determined and the oxygen values of the constituents calculated from the rule given. The following values may be given as examples:

1 cc. *N* formic	acid	reduces	2·00 cc. *N*	dichromate
,, oxalic	,,		1·00	,,
,, acetic	,,		0·00	,,
,, malonic	,,		4·00	,,
,, lactic	,,		4·00	,,
,, succinic	,,		0·00	,,
,, propionic	,,		6·00	,,
,, butyric	,,		12·00	,,
,, valerianic	,,		18·00	,,

(or an increment of 6 cc. *N* dichromate for each CH_2 group).

Amino acids follow the same rule:

1 cc. *N* glycine		reduces	6·00 cc. *N*	dichromate
,, alanine	,,		4·00	,,

By a simple calculation the proportions of two acids in a mixture can be ascertained, provided there is a difference in their oxygen value.

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XCV. THE REARING OF CHICKENS ON THE INTENSIVE SYSTEM. PART III. B-VITAMIN REQUIREMENTS. COMPARISON OF YEAST EXTRACTS AND DRIED YEAST.

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In our previous experiments [1922] it was shown that chickens could be reared from the day of hatching to maturity, under laboratory conditions, if the diet contained sufficient quantities of the three vitamins; if in addition, the protein in the diet were of "good" quality, the birds reached maturity at a rapid rate. The same diet has been used by Miss Malcolm and Mrs Pember on their poultry farm in Sussex and again the rapid growth to maturity with extraordinary health and vigour was observed.

In order to make the results of greater practical value and also to ascertain the real B-vitamin requirements, the ingredients of the diet needed simplification. As a substitute for caseinogen and secwa, dried skim milk is very suitable, since it contains the same substances, caseinogen, lactalbumin and lactose and probably also the same amount of B-vitamin. The replacement of oatmeal by other cereals presents a more difficult problem. Cereal grains contain B-vitamin in different and unknown quantites, so that the amount of marmite needed to balance the carbohydrate will vary for each one. It was orginally supposed that the B-vitamin in oatmeal would be destroyed in the process of kilning used in its preparation, but this required verification; further all samples of oatmeal may not be prepared in the same way. Polished white rice forms the most convenient basal cereal, as it is free from B-vitamin. The amount of B-vitamin necessary to balance this food is likely to suffice for any other cereal. Chick and Hume [1917] have already found that 1 g. of yeast extract is a sufficient daily quantity to prevent polyneuritis in pigeons on a diet of white rice, but it does not follow that this quantity will be adequate for birds of other species. As will be shown later, the requirements of chickens for B-vitamin are greater than those of pigeons.

The determination of the total amount of B-vitamin, which is necessary, reckoned in terms of yeast extract, can only be made if all the constituents of the diet are free from this vitamin. Dried skim milk is almost certain to contain B-vitamin and must be substituted by a protein without it. Such a protein is egg-white. Very little B-vitamin is present in white fish as found by Cooper [1914]. White fish meal in small quantities could therefore be used instead of egg-white.

Marmite as the source of B-vitamin is too expensive for feeding animals. A cheaper form of yeast extract, cerema, has been introduced and dried yeast is another commercial article. The relative values of these varieties of yeast need determination. Marmite has been so frequently used in scientific investigations that it may be taken as the standard for comparison. These experiments have been designed to ascertain:

(1) the relative values of marmite, cerema, and dried yeast as a source of B-vitamin;

(2) the amount of marmite required to balance a diet of white rice and dried skim milk;

(3) the amount of marmite needed to balance a diet of white rice and white fish meal;

(4) by calculation from these data, the amounts of B-vitamin, in terms of marmite, in the foodstuffs used.

Complications could arise from the different amounts of salts, fat and A-vitamin in the diets. These foods contain very little fat, so that a constant supply of fat and A-vitamin could be introduced as cod-liver oil. The salt content of the diets are almost equal. No antiscorbutic was added because there are many observations that birds can exist for a considerable time without C-vitamin. The birds of these experiments have since been kept for over a year without C-vitamin, proving it is not needed (see Part IV, p. 787).

Experimental.

Housing. The housing provided for the chickens has consisted of ordinary commercial appliances. Two houses of each size were procured and placed side by side so that any comparisons, which were required, could be made under the same conditions. The houses were:

(1) three compartment foster mother, or brooder, measuring $9' \times 2'\ 6'' \times 1'\ 6''$, one of the compartments being provided with an extra metal cover and heated by a paraffin lamp;

(2) house measuring $7' \times 5' \times 5'\ 6''$ with run $4' \times 5' \times 3'$;

(3) house measuring $6' \times 4' \times 4'$ with run $4' \times 4' \times 2'\ 6''$ and with three outside nest boxes fitted with trap nests;

(4) house measuring $4' \times 3' \times 4'$ with temporary run $4' \times 4' \times 2'\ 6''$;

(5) house measuring $6' \times 4' \times 4'$ with temporary run $5' \times 4' \times 2'\ 6''$;

(6) fireless brooder measuring $1'\ 4'' \times 1'\ 4'' \times 1'\ 3''$. This was placed in a larger house (2) divided by wooden partitions into two equal parts.

The houses were provided with wooden floors and peat moss litter was used as covering.

No room was available for these houses inside the laboratory. They were placed upon the flat roof of the school building. No food other than that supplied could reach the birds, but in the autumn falling leaves from adjacent trees fell upon the roof and occasionally a few were blown into the runs. In our previous experiments it was found that housing in a room or upon a roof made no essential difference to the experimental results.

The houses provided for pairs of pigeons were double wire show cages for poultry, measuring $4' \times 2' \times 2'\ 3''$ protected at the sides by a metal piece and covered except at the front by wood. Each cage was fitted with a central perch.

Birds. The chicks, generally started as day-old, have been most kindly placed at our disposal by Miss Malcolm and Mrs Pember from their stock at Wellingham poultry farm, Lewes, and have consisted of Light Sussex except in one experiment in which a cross Wyandotte and Light Sussex was used.

Two pairs of pigeons were procured from a dealer.

Diet. The different diets and the results of the feeding are given under the several group headings numbered in continuation of our previous work. In general, the foodstuffs were mixed together as described in our earlier papers.

Comparison of the yeast extracts, marmite and cerema, on pigeons.

In testing foodstuffs for their B-vitamin value pigeons have been the experimental animal. Chick and Hume [1917] found that 1 g. of yeast extract per day was sufficient to prevent polyneuritis on a daily diet of 40 g. of white rice. In our former experiments with chickens 0·5 g. of marmite for 30 g. of oatmeal and milk and 0·25 g. for oatmeal with caseinogen and secwa in the diet was sufficient to prevent leg weakness. It appeared in the case of growing chickens that the carbohydrate of the food needed balancing by marmite. The balancing of the carbohydrate by B-vitamin has been considered necessary by former observers, but it is difficult to prove definitely.

As a preliminary to the comparison of marmite and cerema as a source of B-vitamin for growing chickens, an experiment was started on Feb. 7th, 1922, on two pairs of adult homer pigeons. One pair, red in colour, was fed upon white rice to which were added 0·75 g. of marmite for every 90 g. of rice and 0·75 cc. of cod-liver oil. The other pair, blue in colour, was given the same diet, but 0·75 g. of cerema instead of marmite. Neither pair of birds appeared to like the diet, so that after 14 days the cod-liver oil was omitted. On Feb. 24th, *i.e.* in the third week, both hens showed signs of leg weakness and a high stepping gait and were unable to get upon the perch; both cocks were also unwell. Their feathers were ruffled. Each bird was therefore dosed with 0·25 g. of marmite, or of cerema, dissolved in about 5 cc. of water on two consecutive days. This caused improvement in all the birds. The quantity of marmite, or of cerema, in the food was therefore not sufficient and was raised

on Feb. 27th to 1·5 g. per 90 g. of rice. This additional quantity did not, however, prevent the birds from getting ill again and they were all dosed with 0·5 g. of cerema, or of marmite, for three consecutive days. Their condition improved and their appetite increased. The marmite and cerema were both raised to 3 g. per 90 g. of rice on Mar. 7th.

A diet consisting only of polished rice and marmite, or cerema, cannot be considered adequate without additional protein, salts and A-vitamin. Fish meal (sample 1 see p. 785) was added on Mar. 11th.

On this diet, consisting of 90 g. white rice, 15 g. fish meal and 3 g. of marmite, the red pair of birds improved, gained in weight and the hen laid eggs on Apr. 5th. The blue pair of birds with 3 g. of cerema in the place of marmite did not show a similar improvement: their appetite fell off and they looked unwell with ruffled feathers. They were given a dose of 1 g. cerema on Mar. 27th and at the same time the amount of cerema in the food was raised to 3·75 g. per 90 g. of rice. This amount again did not suffice. The hen bird on Apr. 4th showed the characteristic symptoms of polyneuritis. She recovered after three doses of 1 g. cerema and was able to walk about the next day. On Apr. 12th the cerema was raised to 6 g., and on Apr. 23rd to 7·5 g. per 90 g. rice. The original supply of cerema was now exhausted. The new supply was quite different in appearance and it was learned that the original was from some old stock. The experiment was therefore stopped and both pairs of birds placed on oatmeal and fish meal to ascertain if C-vitamin was needed in their food (see Part IV, p. 787).

Thus, as far as marmite is concerned, 3 g. per 90 g. rice is sufficient to keep pigeons in health. The food consumption was on the average 45 g. rice and 7·5 g. fish meal per day. This is rather more than that given by Chick and Hume.

It may be thought that the salt addition with the fish meal was the reason for the maintenance of the red pair of pigeons, but this does not explain the failure of the blue pair on exactly the same diet.

With insufficient marmite or cerema not only did the appetite fall off, but also the weights. There was an increase of appetite after dosing and increase in weight. A steady increase in weight was shown by the red pair as soon as the balance of rice and marmite was reached. The weights are given in the following table:

	Red pair		Blue pair	
	Cock	Hen	Cock	Hen
	g.	g.	g.	g.
Feb. 9	445	439	434	327
„ 15	435	430	429	318
„ 22	409	391	392	286
Mar. 1	366	392	363	273
„ 8	354	366	347	281
„ 15	368	364	353	269
„ 22	414	414	365	281
„ 29	442	467	368	311
Apr. 7	438	450	407	321
„ 19	447	472	398	284

GROUP IV. *Value of cerema and rice diet.*

Groups I to III of chickens had been kept on oatmeal and milk, or oatmeal and caseinogen and secwa with 0·75 g. of marmite per 90 g. of oatmeal and the results reported in our former papers [1922].

This experiment with Group IV was made to test the value of cerema as a source of B-vitamin for chickens. At the same time white rice was substituted for oatmeal and dried skim milk for caseinogen and secwa.

Twelve day-old Light Sussex chicks were used and the experiment was started on Feb. 22nd, 1922.

The diet was composed of equal parts of white rice and of dried skim milk. A-vitamin was added as cod-liver oil, at first 0·25 cc. but after the tenth day 0·5 cc. per bird per day. B-vitamin was included as cerema in the proportion of 0·75 g. per 90 g. of rice, the same quantity as the marmite used for oatmeal and milk. C-vitamin was not added as it was desired to see if chickens could be reared without it (see Part IV). During the second and third weeks the appetite of the birds was not good and it was not necessary to increase the daily food supply as often as expected. During the fourth week some slight signs of leg weakness were evident. On Mar. 30th at the beginning of the fifth week most of the birds showed very distinct signs of leg weakness. The amount of cerema in the food was then doubled. In addition, each bird was given a dose of 1 g. of cerema dissolved in about 5 cc. of water. This dosing was repeated on Apr. 4th and the amount of cerema in the food raised to 3 g. per 90 g. of rice. After a third dose the birds got slightly better, but still remained weak. Cerema was raised on Apr. 6th to 4·5 g. and on Apr. 11th to 6 g. per 90 g. of rice. The improvement was not maintained in spite of occasional doses of 1 g. of cerema. Ten birds died between Apr. 10th and May 19th. The remaining two recovered after raising the cerema to 7·5 g. per 90 g. of rice and were kept to maturity.

No definite result is given by the experiment, but it indicates that either more B-vitamin is required to balance rice than oatmeal, if cerema and marmite are equivalent, or cerema has less B-vitamin than marmite.

The record of the food consumption and weights of the birds is of no value.

GROUP V. *Value of cerema and oatmeal diet.*

The failure to rear the chickens of Group IV on equal parts of white rice and dried skim milk with the addition of 0·75 g. of cerema per 90 g. of rice led to a return to oatmeal as carbohydrate. Equal parts of oatmeal and dried skim milk give a food mixture of the composition:

Water	Ash	Fibre	Protein	Fat	Carbohydrate
8·1 %	4·7 %	0·5 %	21·9 %	4·3 %	60·6 %

with a protein to fat and carbohydrate ratio of 1 : 3.

This mixture corresponded fairly closely with the original mixture of

5/7 oatmeal, 1/7 caseinogen and 1/7 secwa used in the latter part of the experiment with Group III, namely

Water	Ash	Fibre	Protein	Fat	Carbohydrate
7·0 %	3·7 %	0·7 %	22·4 %	6·1 %	60·2 %

and protein to fat and carbohydrate ratio of 1 : 3.

Cod-liver oil was added to supply A-vitamin, at first at the rate of 0·5 cc., later 1·5 cc. per bird per day. Cerema was added to supply B-vitamin in the proportion of 0·75 g. per 90 g. of oatmeal. Orange juice was included for the first 37 days at the rate of 1 cc. per bird per day; it was afterwards omitted. Ten day-old Light Sussex chicks were used and the experiment started on May 4th, and concluded on Aug. 2nd, 1922.

Except for the accidental loss of five birds during the second week, owing to the paraffin lamp of the brooder smoking, the birds were raised to maturity without failure. The five dead birds were examined and no lesion could be discovered other than the lungs being filled with soot.

The food consumption and growth, owing to the accident, cannot be considered normal during the second and third weeks as all the birds were affected by the soot. The record is as follows:

	Oat-meal	Dried skim milk	Cod-liver oil	Cerema	Orange juice	Average weekly weights:					
	g.	g.	cc.	g.	cc.		g.		g.		g.
May 5	30	30	2·5	0·25	10	May 4	39·4	July 1	806		
„ 6–29	60	60	5·0	0·5	10	„ 10	59·2	„ 12	1099		
„ 30–June 1	90	90	7·5	0·75	5	„ 17	91	„ 19	1282		
June 2–9	120	120	7·5	1·0	5	„ 24	155	„ 27	1471		
„ 10–14	120	120	7·5	1·0	0	„ 31	228	Aug. 2	1648		
„ 15	150	150	7·5	1·25	0	June 7	295				
„ 16–26	180	180	7·5	1·5	0	„ 14	421				
„ 27–July 10	270	270	7·5	2·25	0	„ 21	560				
July 11–Aug. 3	360	360	7·5	3·0	0	„ 28	736				

No comparison of the rate of growth can be made with the birds of Group III which were white Leghorns.

On a carbohydrate basis of oatmeal 0·75 g. of cerema is sufficient per 90 g. This is the same as the quantity of marmite used in Group III. Cerema and marmite thus appear of equal value as a source of B-vitamin, if the amounts of B-vitamin in dried skim milk and in caseinogen and secwa are the same.

GROUP VI. *Comparison of marmite and cerema on chickens.*

The experiment with Group IV had shown that the addition of 0·75 g. of cerema per 90 g. of rice was not enough to maintain the birds. The experiment of Group V with 0·75 g. cerema per 90 g. of oatmeal indicated that this extract had an equal value to marmite. A thorough comparative experiment was now made on two lots of birds simultaneously, the one lot with cerema, as source of B-vitamin, and the other lot with marmite. The experiment with pigeons had shown that 3 g. of marmite per 90 g. of white rice was sufficient. This quantity of each extract was therefore included in the diet. Cod-liver oil to supply A-vitamin was added in the proportion of 0·5 cc. per bird per day. C-vitamin was omitted.

The diet was composed of equal parts of white rice and dried skim milk and had the following composition:

Water	Ash	Fibre	Protein	Fat	Carbohydrate
10·0 %	4·1 %	0·2 %	19·1 %	0·3 %	66·3 %

with a protein to fat and carbohydrate ratio of 1 : 3·5.

The experiment was started on June 26th, and concluded on Dec. 19th, 1922, at which date it was decided to put the hens and one cock into a single group on the diet with marmite for the purpose of procuring a second generation on a C-vitamin free food mixture (see Part IV, p. 787).

The constituents of the diet remained the same throughout; the only alteration was a reduction of the dried skim milk to 1/3 of the total on Aug. 21st (end of 8th week), mainly for the purpose of lessening the cost of feeding. The composition of the food mixture then became:

Water	Ash	Fibre	Protein	Fat	Carbohydrate
10·5 %	2·9 %	0·2 %	14·9 %	0·4 %	71·2 %

with a protein to fat and carbohydrate ratio of 1 : 4·8.

The group consisted of two lots of ten day-old chicks Wyandotte crossed with Light Sussex. Of these twenty birds, only two did not reach maturity. Both belonged to the cerema lot. One died on the 9th day of the experiment; no reason could be found on post mortem examination. The other was found paralysed in one leg at the end of the 9th week; post mortem examination indicated that this was due to pressure by a fibrous ovary on a nerve. On Sept. 28th, at the end of the 13th week, both groups showed signs of slight leg weakness. The birds were always wanting to rest and some of the cocks had a slight high stepping gait. Both the cerema and marmite were then raised, the marmite to 3·75 g. and the cerema to 4·5 g. per 90 g. of rice. The reason for the higher increase of cerema was because the birds of this lot seemed slightly worse. The symptoms disappeared in both lots in about 14 days.

Cocks of each lot started to crow during the 16th week.

On Nov. 11th (20th week) the birds of the cerema lot again showed slight signs of leg weakness. The cerema was then increased to 5·0 g. per 90 g. of rice. This figure was chosen so as to correspond with the nitrogen content of the preparations: marmite 5·7 %, cerema 4·3 %. After raising the cerema the symptoms disappeared.

On Nov. 15th (21st week) it became essential to separate the hens and cocks; the cerema lot became one cock and three hens, the marmite lot one cock and four hens. Excess cocks were kept in their separate lots. The hens of the cerema lot started laying on Dec. 10th (24th week), those of the marmite lot on Dec. 16th.

The total number of hens was too small to continue as two breeding pens. One cock and the seven hens were made into a single group and the experiment as regards comparison of cerema and marmite was terminated. The birds

were kept longer for more evidence about C-vitamin and its non-requirement by chickens (see Part IV, p. 787).

The two yeast extracts appear to be of nearly equal value; the two losses in the cerema lot cannot be fairly attributed to the presence of less B-vitamin. The early loss may have been due to a weaker chick at the start, the later one to some fault in sexual development. 5·0 g. of cerema is the equivalent of 3·75 g. of marmite per 90 g. of white rice with dried skim milk in the food. Throughout, the daily food consumption and the average weekly weights were very similar, as seen from the tables:

Daily food consumption.

	Rice g.	Dried milk g.	Cerema g.	Cod-liver oil cc.
June 26	30	30	1·0	5
„ 27–July 7	60	60	2·0	5
July 8–12	120	120	4·0	5
„ 13–20	150	150	5·0	5
„ 21–23	180	180	6·0	5
„ 24	240	240	8·0	5
„ 25–Aug. 2	270	270	9·0	5
Aug. 3–6	300	300	10·0	5
„ 7–8	330	330	11·0	5
„ 9–11	360	360	12·0	5
„ 12–13	390	390	13·0	5
„ 14–20	420	420	14·0	5
„ 21–Sept. 6	570	285	19·0	5
Sept. 7–25	780	390	26·0	5
„ 26–27	840	420	28·0	5
„ 28–30	840	420	35·0	5
Oct. 1–24	840	420	42·0	5
„ 25–31	900	450	45·0	5
Nov. 1–10	960	480	48·0	5
„ 11–Dec. 17	960	480	52·5	5

	Rice g.	Dried milk g.	Marmite g.	Cod-liver oil cc.
June 26	30	30	1·0	5
„ 27–July 7	60	60	2·0	5
July 8–12	120	120	4·0	5
„ 13–19	150	150	5·0	5
„ 20	180	180	6·0	5
„ 21–23	240	240	8·0	5
„ 24–31	270	270	9·0	5
Aug. 1–2	300	300	10·0	5
„ 3–8	330	330	11·0	5
„ 9–11	360	360	12·0	5
„ 12–13	390	390	13·0	5
„ 14–16	420	420	14·0	5
„ 17–20	450	450	15·0	5
„ 21–Sept. 6	600	300	20·0	5
Sept. 7–25	810	405	27·0	5
„ 26–27	870	435	29·0	5
„ 28–Oct. 24	870	435	36·25	5
Oct. 25–31	930	465	38·75	5
Nov. 1–8	990	495	41·25	5
„ 9–Dec. 17	1050	525	43·75	5

Average weekly weights.

	Cerema lot g.	Marmite lot g.
June 26	40	40
July 3	76	75
„ 10	132	131
„ 17	215	200
„ 24	282	283
„ 31	431	390
Aug. 7	549	498
„ 10	628	579
„ 21	845	797
„ 28	988	972
Sept. 4	1140	1123
„ 11	1279	1307
„ 25	1595	1653

	Cerema lot		Marmite lot	
	Cocks g.	Hens g.	Cocks g.	Hens g.
Oct. 2	1927	1479	2062	1426
„ 9	2143	1594	2302	1572
„ 16	2351	1688	2486	1659
„ 23	2424	1713	2615	1700
„ 31	2701	1815	2876	1847
Nov. 8	2732	1872	2983	1931
„ 15	2862	1952	3032	1986
„ 22	2930	1985	3055	1993
Dec. 4	3070	2082	3198	2030
„ 11	3108	2225	3240	2090
„ 18	3192	2346	3288	2141

GROUP VII. *Comparison of cerema and dried yeast.*

Two lots of ten day-old Light Sussex chicks were used for this comparison, which was started on Dec. 22nd, 1922. The food for each lot consisted of two-thirds white rice and one-third dried skim milk. Cod-liver oil was added to each daily food supply at the same rate as before, 5 cc. per day, or 0·5 cc. per bird. C-vitamin was omitted. One lot received cerema in the proportion of 6 g. per 90 g. of rice. This quantity was chosen so as definitely to avoid any signs of leg weakness and was rather greater than the amount of 5 g. previously found to be sufficient. The other lot received 6 g. of dried yeast per 90 g. of rice. The two diets are not exactly equivalent on account of the higher nitrogen or protein content of dried yeast, 7·6 % against 4·3 % of cerema. This was unavoidable, as no similar protein could be added to compensate.

On Jan. 16th, 1923, the birds of the dried yeast lot showed distinct signs of leg weakness and drooping wings. The dried yeast was therefore raised to 12 g. per 90 g. of rice. On Apr. 6th, in the 15th week of the experiment, some signs of leg weakness became noticeable in the cerema lot; the amount of cerema was raised to 6·75 g. per 90 g. of rice. No other alteration was made and the experiment was terminated on Apr. 14th. Cocks of each group began to crow about Mar. 25th. The birds were mature and the hens needed separation from the cocks. The hens and a cock were continued as a single group for ascertaining egg production and hatchability on the C-vitamin-free diet (see Part IV). The first egg was laid on Apr. 29th in the 20th week.

Daily food consumption.

	Rice g.	Dried skim milk g.	Cerema g.	Cod-liver oil cc.
Dec. 22–27	60	30	4	5
„ 28	90	45	6	5
„ 29–Jan. 7	120	60	8	5
Jan. 8–9	150	75	10	5
„ 10–11	180	90	12	5
„ 12–14	210	105	14	5
„ 15–19	240	120	16	5
„ 20–24	270	135	18	5
„ 25–29	300	150	20	5
„ 30–31	330	165	22	5
Feb. 1–7	360	180	24	5
„ 8–9	390	195	26	5
„ 10–13	420	210	28	5
„ 14	450	225	30	5
„ 15–19	480	240	32	5
„ 20	510	255	34	5
„ 21–23	540	270	36	5
„ 24–26	600	300	40	5
„ 27–Mar. 6	660	330	40	5
Mar. 7–Apr. 5	720	360	48	5
Apr. 6–13	720	360	54	5

	Rice g.	Dried skim milk g.	Dried yeast g.	Cod-liver oil cc.
Dec. 22–25	60	30	4	5
„ 26–28	90	45	6	5
„ 29–Jan. 2	120	60	8	5
Jan. 3–7	150	75	10	5
„ 8–9	180	90	12	5
„ 10–11	210	105	14	5
„ 12–14	240	120	16	5
„ 15–17	270	135	36	5
„ 18–19	300	150	40	5
„ 20–22	330	165	44	5
„ 23–25	360	180	48	5
„ 26–29	390	195	52	5
„ 30–31	420	210	56	5
Feb. 1–19	450	225	60	5
„ 20	480	240	64	5
„ 21–23	540	270	72	5
„ 24–26	600	300	80	5
„ 27–Mar. 6	660	330	88	5
Mar. 7–30	720	360	96	5
„ 31–Apr. 13	780	390	104	5

Altogether three birds out of 20 were lost. One bird of the cerema lot died on the second day of the experiment. Another bird of this lot dislocated

its leg in the second week and was chloroformed. The third bird belonged to the dried yeast group and was found dead on Feb. 6th in the 7th week; it had suffered more severely than the other birds from insufficiency of dried yeast before the quantity was raised and owing to its weakness may have been crushed by the birds huddling together at night. Excluding the accidental loss, 90 % of each lot was raised to maturity. Just before the conclusion of the experiment, on Apr. 7th, a cock of the cerema lot was found with a damaged leg, evidently from fighting and was chloroformed.

The close parallelism between the two lots is shown by the daily food consumption (p. 780) and the weekly average weights of the birds which were:

	Cerema lot g.	Dried yeast lot g.		Cerema lot g.	Dried yeast lot g.
Dec. 22	37·6	36·7	Feb. 23	921	992
„ 29	76·6	74·8	Mar. 2	1072	1089
Jan. 5	132·4	137·6	„ 9	1262	1334
„ 12	220	234	„ 16	1418	1508
„ 19	284	309	„ 23	1596	1598
„ 26	386	400	„ 30	1801	1840
Feb. 2	514	502	Apr. 6	1920	2052
„ 9	604	665	„ 13	2034	2144
„ 16	748	810			

The higher consumption in the dried yeast lot was due to its having one or two more birds at certain periods, as the result of losses in the cerema lot. The food consumption per bird is approximately the same in both lots.

The advantage, if any, is on the side of the dried yeast lot. The two lots contained the same number of cocks and hens.

It is interesting to note that these weights run very close to the weights of the birds in Group VI. Group VI was started in June, Group VII in December. There is no appreciable difference in the rate of growth at different seasons of the year, as is commonly believed by poultry keepers. The result shows that a proportion of 12 g. of dried yeast is equal to 6, or more probably, 6·75 g. of cerema per 90 g. rice on a diet of two-thirds white rice and one-third dried skim milk.

Groups VIII and IX. *Amount of marmite required for a diet containing no B-vitamin.*

In order to determine the full amount of marmite which must be included in a diet not containing B-vitamin in any of its ingredients, it was necessary to replace the dried skim milk in the diet of the previous groups by some protein with little or no B-vitamin. Cooper [1914] has shown that more than 10 g. per day of white fish was required to prevent polyneuritis in pigeons. The total amount necessary was not determined, but this result suggests that white fish flesh has very little B-vitamin. Dried commercial white fish meal thus seemed a suitable protein for these experiments, if used in small amounts. 5 g. of fish meal with 90 g. of rice would not be likely to introduce an appreciable amount of B-vitamin.

Fish meal contains a large proportion of mineral matter, so that a further ash supplement is unnecessary. Some samples of fish meal have a low fat content (see p. 785 for analyses) and the amount of A-vitamin in this fat is probably small. Fish meals, dark in colour, are evidently made at a high temperature and some, if not all, of the A-vitamin would be destroyed. A known amount of fat supplying A-vitamin in the form of cod-liver oil was therefore added.

The quantity of marmite to be included as source of B-vitamin required consideration. Group VII showed that 6 g. of cerema or 12 g. of dried yeast was adequate for 90 g. of rice and 30 g. of dried skim milk. This amount was likely to be too small on the fish meal diet, since the dried skim milk probably carried enough B-vitamin to balance its carbohydrate, fat and protein. The dried skim milk supplied 28 parts of combustible food material and the rice supplied 58 parts. Half as much cerema again would be required to balance the 28 parts derived from the dried skim milk, now replaced by fish meal and a proportion of rice. Consequently for the fish meal diet containing 90 g. of rice and 5 g. of fish meal $6 + 3 = 9$ g. of cerema would be needed. Cerema and marmite are nearly equal, so 9 g. of marmite was added to balance these amounts and a small allowance of cod-liver oil, 0·5 cc. per 90 g. of rice.

The diet composed of 90 g. of rice, 5 g. of fish meal (sample 2), 0·5 cc. of cod-liver oil and 9 g. of marmite had the composition:

Water	Salt	Ash	Fibre	Protein	Fat	Carbohydrate
12·7 %	1·7 %	2·7 %	0·3 %	11·3 %	1·0 %	70·6 %

with a protein to fat and carbohydrate ratio of 1 : 6·3. Marmite is present in the proportion of 8·6 %.

In order to ascertain if the amount of marmite was properly adjusted, the cod-liver oil was raised at intervals to 1·0, 1·5, 2·0, 3·0, 4·0, 5·0 cc. At the same time the effect of the extra cod-liver oil and its relation to the amount of B-vitamin would be shown. Our previous experiments (Part I) had indicated that extra cod-liver oil needed balancing with B-vitamin. This question is being further studied in another group of birds.

Group VIII consisted of 11 Light Sussex birds, 7 weeks old, previously kept on a rice and dried skim milk diet as used for Group VII. The experiment was started on Mar. 9th and concluded on June 21st, 1923 (14 weeks). The birds did not grow rapidly and there was considerable difference in the weights of the birds of the same age and sex, particularly noticeable after the oil was raised to 2·5 and 4 cc. Some of the hens became adult long before others. One of them had a sort of spinal curvature. The birds were not active and always wanted to rest. Signs of leg weakness with high stepping gait and drooping wings were observed. Their feathering was slow and their combs were pale. The cocks were never heard to crow. All the birds were raised to maturity. The general appearance of the birds was the best indication that the diet contained just the sufficient amount of B-vitamin to maintain them and take them to maturity, but not enough to keep them in

good health. The conditions of the experiment were repeated in the next group.

Group IX consisted of 10 Light Sussex day-old chicks, which were fed on the same diet as Group VIII. The experiment was started on Apr. 7th and concluded on Aug. 24th, 1923 (20 weeks).

Between May 4th and June 19th, the cod-liver oil was gradually raised from 0·5 cc. to 4 cc. (see table of weights). With the increase of the cod-liver oil the birds had the appearances of those of Group VIII, pale combs, slight leg weakness, high stepping gait, poor feathering, which pointed to an insufficiency of B-vitamin in the diet. The marmite was therefore raised on June 30th (13th week) to 9·75 g. per 90 g. rice. Some improvement followed. It was raised again on Aug. 3rd (17th week) to 10·5 g. per 90 g. of rice. The general improvement was more noticeable.

The food intake was not measured daily as in other groups. The food mixture was made up in sufficient quantity to last for two or three days. The birds were allowed as much as they would eat; food was constantly before them. The actual consumption has no particular bearing on the observations.

Three birds were lost during the experiment. One was unwell on arrival and died the first day. The second died on June 20th. This bird had the most severe symptoms of leg weakness in the group. Post mortem examination showed considerable abnormality of the gut, the walls of which were very thick. The third died on Aug. 7th. This bird had stoppage and distention of the gut at the junction of the caeca. The connective tissue holding the intestines was very tight and fibrous.

One of the birds raised to maturity showed some deformity in one wing, probably from resting on it during the time the leg weakness was observed.

It is not possible to compare the growth with that of the birds of Groups VI and VII, which had not only more protein, but also milk protein in the diet. In order to see the irregularity the weekly weight record of each bird must be recorded (see p. 784).

The weekly weights are not the best indication of the condition of the birds. The condition can really only be judged by comparison with other birds which have been reared on a diet containing all the constituents in better proportions. At 20 weeks the birds were not mature. The cocks never crowed and the hens were small for their age. It was difficult to be certain of the sex of two of the birds, Nos. 2 and 6. Only at the end did their sex become quite certain.

The results confirm those of Group VIII. On a diet of 90 g. rice, 5 g. fish meal and 0·5 cc. cod-liver oil, 9 g. of marmite is sufficient to maintain and raise them to maturity. Excess of cod-liver oil produced signs of leg weakness. These observations do not agree with those of Emmett and Peacock [1923] who attribute leg weakness and drooping wings to lack of A-vitamin. A large excess of cod-liver oil was present in the diets of Groups VIII and IX with scarcely enough marmite to balance the rice and fish meal adequately. Our

result suggests that the diet used by Emmett and Peacock had not enough B-vitamin in it. Perfect health and growth depend upon a proper amount of all the constituents in the diet. The question of the balance of carbohydrate, fat and protein with B-vitamin is now under investigation.

Average weekly weights, and time of increase of cod-liver oil and marmite.

	Apr. 7	Apr. 13	Apr. 20	Apr. 27	May 4	May 11	May 18	Sex
Cod-liver oil	0·5 cc.	0·5 cc.	0·5 cc.	0·5 cc.	1 cc.	1 cc.	2 cc.	
Marmite	9·0 g.	9·0 g.	9·0 g.	9·0 g.	9·0 g.	9·0 g.	9·0 g.	
No. 1	41·5 g.	59·5 g.	95·0 g.	150 g.	205·0 g.	303·0 g.	385 g.	Cock
,, 2	41·0	66·0	82·0	123	182·0	230·0	317	Cock
,, 3	41·0	62·5	83·0	125	152·0	221·0	293	Cock
,, 4	42·5	63·5	84·0	129	173·0	242·0	329	Hen
,, 5	39·0	61·5	82·0	117	157·0	219·0	292	Hen
,, 6	38·5	59·5	84·0	115	149·0	202·0	250	Cock
,, 7	40·0	53·5	64·0	88	111·0	159·0	203	Hen
,, 8	40·5	63·5	81·0	109	139·0	190·0	238	
,, 9	38·5	49·5	58·0	80	97·0	134·0	184	
,, 10	41·0	Died						
Average	40·3	59·7	79·9	115	151·5	211·0	277	
Increase	—	19·4	20·2	35	36·5	59·5	66	

	May 25	June 1	June 8	June 15	June 22	June 29	July 6	Sex
Cod-liver oil	2·5 cc.	2·5 cc.	2·7 cc.	3·5 cc.	4·0 cc.	4·0 cc.	4·0 cc.	
Marmite	9·0 g.	9·0 g.	9·0 g.	9·0 g.	9·0 g.	9·75 g.	9·75 g.	
No. 1	519 g.	580 g.	800 g.	905 g.	1140 g.	1325 g.	1526 g.	Cock
,, 2	398	520	682	802	935	1100	1241	Cock
,, 3	393	555	675	823	975	1165	1314	Cock
,, 4	429	525	635	777	957	1105	1257	Hen
,, 5	365	495	605	690	840	915	1055	Hen
,, 6	334	425	505	603	680	765	863	Cock
,, 7	253	330	352	422	488	555	612	Hen
,, 8	312	417	498	568	665	775	854	
,, 9	234	300	402	517	Died			
Average	360	494	573	678	835	963	1080	
Increase	83	134	79	105	157	128	117	

	July 13	July 20	July 27	Aug. 3	Aug. 10	Aug. 17	Aug. 24	Sex
Cod-liver oil	4·0 cc.	4·0 cc.	4·0 cc.	4·0 cc.	4·0 cc.	4·0 cc.	4·0 cc.	
Marmite	9·75 g.	9·75 g.	9·75 g.	10·5 g.	10·5 g.	10·5 g.	10·5 g.	
No. 1	1640 g.	1900 g.	1940 g.	2170 g.	2250 g.	2450 g.	—	Cock
,, 2	1312	1480	1605	1730	1940	2100	—	Cock
,, 3	1410	1515	1600	1800	1950	2025	—	Cock
,, 4	1207	1400	1560	1725	1890	1960	—	Hen
,, 5	1127	1198	1280	1420	1570	1675	—	Hen
,, 6	920	1012	1122	1235	1325	1480	—	Cock
,, 7	659	700	725	800	990	995	—	Hen
,, 8	922	919	857	905	Died			
Average	1149	1265	1336	1473	1702	1812		
Increase	69	118	71	137	229	110		

The comparative B-vitamin value of foodstuffs.

The comparative value of several foodstuffs in preventing polyneuritis in pigeons has been determined by Chick and Hume [1917] and Cooper [1914]. Yeast extract (marmite) was found to contain more B-vitamin than any of the others except rice germ. The B-vitamin content of marmite has also been found to be very constant. The constancy has again been shown by our experiments with pigeons (p. 774). For purposes of comparison, marmite may be chosen as the most convenient standard. If the figure of 1 g. found by

the above observers be put at 100, their figures give the following comparative numbers:

Marmite	100	Ox liver	33	Ox heart muscle	20
Wheat germ	67	Barley, unhusked	27	Ox brain	17
Pressed yeast	40	Barley, husked	20	Sheep brain	8
Lentils	33	Peas	20	Beef muscle	5
Egg yolk	33				

Groups VI and VII showed that 1·25 g. marmite were equal to 1·66 g. of cerema and 2 to 2·25 g. of cerema were equal to 4 g. of dried yeast. Hence, if marmite = 100, cerema = 75, dried yeast = 50.

Groups VIII and IX were raised to maturity on the diet of 90 g. of rice and 5 g. of fish meal with the addition of 9 g. of marmite, or 8·6 % of the total food.

Group VI, on a diet of equal parts of rice and dried skim milk, needed an addition of 3 g. of marmite per 90 g. of rice. Assuming that 90 g. of dried skim milk require 9 g. of marmite, as only 3 g. were wanted, this quantity of dried skim milk would contain the equivalent of 6 g. of marmite, or 6·7 %.

It is not possible to calculate from the diet of Group V, equal parts of oatmeal and dried skim milk, the B-vitamin value of oatmeal; but in a further experiment (Group XI, see Part V) chicks were raised to maturity on a diet of 90 g. of oatmeal and 5 g. of fish meal with an addition of 1·5 g. of marmite. The 95 g. of food would thus contain an equivalent of 9 − 1·5 = 7·5 g. of marmite or 7·9 %.

Oatmeal, as a cereal, thus contains a considerable amount of B-vitamin, but it is not sufficient for the rearing of chicks. It does not follow that whole oats contain the same amount, nor that other cereals and cereal products have an equal amount. Germ meals may contain more. Further feeding experiments will be necessary to ascertain the amounts. In the meanwhile, in order to be on the safe side for maintaining the health of chicks, the addition of some foodstuff rich in B-vitamin is advisable. The maximum needed would be 8·6 % in terms of marmite or 17 % in terms of dried yeast. With a cereal such as oatmeal an addition of 1·5 % of marmite or 3 % of dried yeast would be adequate.

Analyses of foods.

The foods which have been used in these experiments had the following composition, from which the composition of the actual diets have been calculated:

	Water %	Salt %	Other ash %	Fibre %	Protein %	Fat %	Carbohydrate By diff. %	Carbohydrate Undet. %
Oatmeal*	7·5	—	1·9	1·0	12·0	8·4	69·4	—
Rice white*	11·4	—	0·6	0·3	6·5	0·4	80·8	—
Dried skim milk (1)	7·0	—	7·6	—	31·8	0·1	43·3†	10·2
„ „ „ (2)	8·6	—	7·6	—	31·8	0·2	43·4†	8·4
Fish meal (1)	14·7	3·1	18·6	—	60·5	3·3	—	—
„ „ (2)	12·1	3·2	21·3	—	59·8	1·9	—	—
Marmite	27·3	14·5	13·9	—	32·5	—	11·8	—
Cerema	28·6	6·9	10·5	—	24·4	—	29·6	—
Dried yeast	2·5	—	8·6	—	43·5	0·9	44·5	—

* From *Analyses and Energy Values of Foods* by R. H. A. Plimmer. † Lactose by analysis.

SUMMARY.

1. Pigeons on a diet of polished rice (90 g.) and fish meal (15 g.) need an addition of 3 g. of marmite for maintenance.

2. Chicks on a diet of equal parts of white rice and dried skim milk need an addition of 3 g. of marmite per 90 g. of rice, or 3·3 %.

3. Chicks on a diet of equal parts of oatmeal and dried skim milk need an addition of 1·5 g. of marmite per 90 g. of oatmeal, or 1·6 %.

4. Chicks on a diet of 90 g. of rice and 5 g. of fish meal with 0·5 cc. of cod-liver oil need an addition of 9 g. of marmite, or 8·6 % of the food.

5. If marmite be put at 100, cerema has a value of 75, dried yeast of 50, oatmeal of 7·9, dried skim milk of 6·7.

We gratefully acknowledge supplies of marmite, cerema and dried yeast from the Marmite Food Extract Co., Ltd.

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XCVI. THE REARING OF CHICKENS ON THE INTENSIVE SYSTEM. PART IV. C-VITAMIN REQUIREMENTS OF CHICKENS AND OTHER BIRDS.

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The growth of the chick in the shell to the day of hatching is one of the best indications that C-vitamin is not needed by the growing bird, since there is no evidence to show that the contents of eggs have any anti-scorbutic value. Further, in those species of animals which suffer from scurvy, the young are most quickly affected. These considerations suggested to us that birds would not require C-vitamin at any time during their lives. The earlier workers upon beri-beri maintained chickens for many weeks upon a diet free from C-vitamin. More recently, Shorten and Ray [1921] kept fowls for 13 weeks on a diet of dried vegetables, proved by experiments on guinea-pigs to be free from C-vitamin, but their birds died from some unknown causes.

Sugiura and Benedict [1923] have kept pigeons on a diet without C-vitamin for 147 days. They consider also that A-vitamin is not required by pigeons (or at most only a very small amount). The birds produced eggs and raised their young on the same diet. Chickens have been kept by Mitchell, Kendall and Card [1923] for about 20 weeks on a diet not containing C-vitamin; the addition of food with C-vitamin to a control set of birds made no appreciable difference to the rate of growth. These observers, and also Emmett and Peacock [1923], find that A-vitamin is needed in considerable amount by chickens.

In view of the fact that scurvy, which is due to the absence of C-vitamin, does not appear in man for a period of four to six months, though guinea-pigs succumb in three to four weeks, a period of 20 weeks can hardly be considered sufficiently long to prove that chickens do not need C-vitamin. At any rate birds should be maintained on such a diet for twice as long as the fatal period in man, *i.e.* for 8 to 12 months. This long period was designed for our experiments and in that time it was expected to raise another generation. The

experiments have been extended to pigeons and other birds. Though success in raising a new generation has been very limited, which appears to be connected with some other deficiency in the diet, it may still be concluded that chickens, pigeons and other birds never seem to require the addition of C-vitamin to their food. The addition of green food to the food of birds so constantly advised by poultry keepers is not for the C-vitamin which it contains.

Experimental.

Chickens.

The birds used in these experiments were those of Group VI and also Group VII. The pigeons were those also described in Part III [1923]. The other birds were ducks, geese, guinea-fowl, turkeys and pheasants. With these latter birds the main experiment was in connection with their comparative B-vitamin requirements (see Part V, p. 794).

Group VI. The chickens of this group were formed into a breeding group on Dec. 19th, 1922, and kept until Aug. 10th, 1923, at which date the experiment was concluded. Throughout the whole period lasting for nearly 14 months these birds were given the same diet of white rice and dried skim milk supplemented with marmite and cod-liver oil.

Eggs were laid regularly from Dec. 1922 to Aug. 1923. The egg record is not of any value, since many eggs were laid in the house, or run, and were eaten by the birds. In order to stop the egg eating, the amount of marmite in the diet was raised on Jan. 15th to 4·5 g., on Feb. 1st to 5·25 g., on Feb. 16th to 6 g., on Mar. 13th to 6·75 g. per 90 g. of rice or 120 g. of food. This did not altogether stop the egg eating.

The hatchability of the eggs was not tested till Feb. 5th. Those laid between this date and Mar. 7th were not fertile. Most of the eggs laid between Mar. 7th and Apr. 9th were unfertile, but five had germs; these were not strong enough to hatch.

The A-vitamin content of the eggs was now investigated by Miss Coward and Mr Channon, whose results will be published shortly. The A-vitamin content was found to be below normal. In consequence of this deficiency the amount of cod-liver oil in the diet of the birds was raised from 0·5 to 2 cc. per bird per day on Apr. 18th.

Eggs tested at the end of April and beginning of May showed greater fertility, but still did not hatch. Many of them had well developed chicks. This ill success in hatching may have been due in part to the incubator, which was of the laboratory pattern not adapted for hatching eggs.

It became possible to test the hatchability of eggs laid in June under a broody hen. Out of three eggs tested one hatched out and has been reared successfully to the middle of August. The other two eggs were not fertile and were laid by a hen whose eggs had throughout been unfertile.

At the same time as these eggs were put under a broody hen, two eggs from a hen of Group VII were also tried; both were fertile and chicks hatched. A second generation has thus been procured from birds whose diet has not contained C-vitamin. As Group VII had given eggs from which birds could be hatched it became unnecessary to keep the birds of Group VI after they had been on a diet free from C-vitamin for about 14 months. The group was therefore got rid of.

During the course of the experiment one of the hens was found dead on inspection in the morning. No actual cause could be found on post mortem examination. An egg was found fully formed in the oviduct and death was probably due to its being egg bound.

Two hens died in July. One of them, an excellent egg layer, had its legs broken probably by the cock and evidently died of the shock. The other never laid an egg; like the hen which died at the end of the ninth week it was found to have a fibrous ovary.

Two other hens were bad egg layers. One laid a few eggs in January. The other, as far as we were able to notice, never laid an egg, though she may have laid in the run and eaten it. Post mortem examination of these hens showed in each case a well developed ovary with eggs in the several stages of development. Both the bodies were covered with fat and in the abdomen there were masses of fat weighing in each case over 500 g. The fat was quite white, owing to the colourless diet of rice and dried skim milk. This fat will have been formed from the carbohydrate and protein of the food which contained only 0·5 % of fat.

As was to be expected from the work of Palmer and Kempster [1919] the yolk of the eggs was colourless. Such white yolks were not observed by them nor by us in the eggs of the birds of Groups I to III. The whiteness of the yolks was best seen after hard boiling; it was then difficult to distinguish between white and yolk. Colour of yolk and reproduction have thus no relationship. The yolk of one of the eggs was analysed and found to correspond with the normal:

	Water %	Ash %	Protein %	Fat %
White yolk	44·4	1·8	15·8	36·7
Normal yolk	47·1	2·0	15·5	33·3

The palatability of the eggs was not different from that of the ordinary egg with yellow yolk.

The chicks hatched from these white yolk eggs were almost pure white, instead of the usual yellowish colour.

The size of the eggs on the average was normal; they seldom exceeded 2 oz., in general they averaged about $1\frac{3}{4}$ oz., or 50 g.

It might appear from these experiments that hatchability was largely connected with the A-vitamin content of the food. This is probably only one factor of that problem; it is much more likely that protein, B-vitamin and proper salts are also concerned.

Group VII. The birds of this group were made into a breeding pen on Apr. 14th, 1923 (see Part III). After one week on the former diet of white rice, dried skim milk, dried yeast and cod-liver oil, the diet was changed to the simplest possible. It was composed of 100 g. rice, 20 g. dried yeast, 0·25 g. of salt and 1 g. of quick lime, slaked with water before being added to the food. The quantity of dried yeast was chosen as 20 g. as it had previously been found that 8·6 % of marmite = 17·2 % of dried yeast was a sufficient addition for 90 g. of rice and 5 g. of fish meal. This diet had the composition:

Water	Salt	Ash	Fibre	Protein	Fat	Carbohydrate
9·8 %	0·2 %	2·7 %	0·2 %	12·5 %	0·5 %	74·0 %

The lime was added as it was found, on analysis, that dried yeast contained only traces of calcium.

Cod-liver oil to supply A-vitamin was omitted from the food up to the end of June, but the birds were given cod-liver oil by hand at the rate of 1 cc. per day for periods of five to seven days. The cod-liver oil was not given to any of the birds until May 15th, *i.e.* until they had been on the new diet for three weeks, at which period several of the birds were unwell. It was then given to the cock and two hens. The effect was striking. The cock who had lost his voice, again began to crow and became very active. The hens which had ceased to lay once more began laying. The dosing of these birds with cod-liver oil was then stopped and two other hens were given the cod-liver oil. They also began to lay after the doses of oil. These remarkable observations require repetition on another set of birds. They were not continued at the time, as birds from another group were mature and were added to this group as a breeding group, and as it was desired to ascertain the egg production and hatchability on this simple diet. Cod-liver oil was added to the food at the rate of 2·5 cc. per 100 g. of rice. The composition was not materially changed except as regards the fat as seen from the following figures:

Water	Salt	Ash	Fibre	Protein	Fat	Carbohydrate
9·6 %	0·2 %	2·7 %	0·2 %	12·3 %	2·5 %	72·4 %

with a protein to fat and carbohydrate ratio of 1 : 6·1.

The results of this diet as regards fertility and hatchability of the eggs will not be known for some time.

At the time of change to this diet on July 1st the birds had been kept without C-vitamin for six months. The eggs laid between Apr. 29th and June 30th were tested for fertility and hatchability. The first eggs up to May 8th were all unfertile. Two eggs laid in June were put under a broody hen and both hatched out and both chicks were thriving well in August. The other eggs were incubated. They were mostly fertile and had well developed chicks; the ill success in hatching was probably due to the incubator being

not specially adapted for hatching eggs. C-vitamin thus appears to be not needed by the chicken at any period of its life.

Pigeons.

The two pairs of pigeons (see Part III) previously kept on a rice and fish meal diet for the comparison of marmite and cerema were placed, on Apr. 25th, 1922, on a diet consisting of 90 g. oatmeal, and 15 g. fish meal (1). This diet had the composition:

Water	Salt	Ash	Fibre	Protein	Fat	Carbohydrate
8·6 %	0·5 %	4·3 %	0·9 %	18·9 %	7·7 %	59·5 %

B-vitamin was added to the extent of 0·75 g. of marmite for the red pair, and 1·5 g. of cerema for the blue pair. Cod-liver oil was not added, as it was probable that sufficient A-vitamin was present in the fish meal. The quantity of cerema was reduced to 0·75 g. 17 days after the start so that both pairs had then the same diet. C-vitamin was omitted.

The daily food consumption was on the average 45 g. oatmeal and 7·5 g. fish meal for each pair. It was greater during the periods of rearing young birds.

Both pairs of birds laid eggs every month during the breeding season, but except once in 1922 and once up to June 1923 no young lived longer than 10 to 14 days. The data of the eggs and rearing is given in the table. On account of the failure to rear the young birds, the diet was slightly altered:

(1) The marmite was omitted from the food of the red pair on June 18th, 1922; the cerema from that of the blue pair on July 13th, 1922.

(2) The fish meal was reduced from 15 to 10 g. on June 28th and to 5 g. on Aug. 1st for both pairs.

This diet consisting only of 90 g. oatmeal and 5 g. fish meal (1) was continued without alteration until June 10th, 1923. Its composition was

Water	Salt	Ash	Fibre	Protein	Fat	Carbohydrate
7·9 %	0·2 %	2·7 %	0·9 %	14·5 %	8·2 %	65·8 %

with a protein to fat and carbohydrate ratio of 1 : 5·1.

The alterations were made with the idea of diminishing the frequency of the egg laying. The failure to rear was possibly due to the desire of the hen bird to lay, as eggs appeared a few days after the young were apparently deserted. The alterations made no difference; one young bird was raised by the red pair from the last eggs of the 1922 season. The red pair again raised one young bird early in the 1923 season; this bird had crooked feet and was chloroformed. At the same time the observation was made that the parent birds did not actually desert their young. The cause of the failure was probably in connection with the diet; it is being sought for by alteration in B- and A-vitamin content. This has consisted in the addition of 1·5 g. of marmite to the food of the red pair and of the addition of 1 cc. of cod-liver oil to that of the blue pair per 90 g. of oatmeal. The effect has been the rearing of another

pair of young by the red pair, but the continued failure of the blue pair. Further experiments on this finding must await the next breeding season.

The maintenance of the two pairs in health for one year and two months, with the production of fertile eggs and at any rate three perfect young birds, is sufficient evidence that pigeons never need the inclusion of C-vitamin in their diet.

An unfortunate accident happened on Nov. 15th, 1922. The two blue birds escaped during the cleaning of their cage. The hen returned the same evening but the cock was lost. Luckily the single red pigeon which had been reared was a cock and he was now paired with the blue hen. All the data of the blue pair since Nov. 20th, 1922 refer to this red cock and blue hen. The red cock had been reared entirely without C-vitamin and the eggs produced were fertile (see table).

Table of egg production by pigeons.

Red pair				Blue pair			
Eggs laid on	Weights g.	Hatched	Result	Eggs laid on	Weights g.	Hatched	Result
15. v. 22	20·4	—	Unfertile	25. v. 22	19·0	11. vi. 22	Both squabs
16. v. 22	22·3	3. vi. 22	Squab died	26. v. 22	19·0	12. vi. 22	died
15. vi. 22	23·1	4. vii. 22	Squab died	24. vi. 22	19·0	12. vii. 22	Both squabs
—	—	—	—	26. vi. 22	19·5	13. vii. 22	died
18. vii. 22	23·2	5. viii. 22	Squab died	25. vii. 22	19·0	11. viii. 22	Both squabs
19. vii. 22	24·7	5. viii. 22	,,	26. vii. 22	20·0	12. viii. 22	died
23. viii. 22	22·2	9. ix. 22	Reared	25. viii. 22	19·2	11. ix. 22	Both squabs
24. viii. 22	24·0	9. ix. 22	Died	26. viii. 22	21·0	12. ix. 22	died
				27. ix. 22	18·0	14. x. 22	Both squabs
				29. ix. 22	19·4	15. x. 22	died
9. iii. 23	22·0	Eggs deserted, addled		6. ii. 23	19·5	Unfertile	—
11. iii. 23	23·0	27. iv. 23	—	8. ii. 23	18·0	,,	—
8. iv. 23	22·5	Reared, crooked feet		24. iii. 23	20·5	7. iv. 23	Both squabs
10. iv. 23	23·5	—	Squab died	26. iii. 23	20·5	7. iv. 23	died
				18. iv. 23	18·5	6. v. 23	Both squabs
				20. iv. 23	19·5	7. v. 23	died
12. v. 23	23·0	29. v. 23	Both squabs	15. v. 23	19·0	3. vi. 23	Squab died
13. v. 23	?	30. v. 23	died	16. v. 23	Broken	—	—
13. vi. 23	23·0	2. vii. 23	Reared	11. vi. 23	20·0	29. vi. 23	Deserted
15. vi. 23	23·0	2. vii. 23	,,	12. vi. 23	20·0	29. vi. 23	,,

The last two eggs were laid after changing the diets, by adding marmite to that of the red pair, cod-liver oil to that of the blue pair.

The weights of the birds remained almost constant. Those of the blue pair rose at first after the recovery from the rice diet. The reduction after Jan. 1923 may be connected with the breeding season. The record was:

	Red cock g.	Red hen g.	Blue cock g.	Blue hen g.
25. iv. 22	434	455	370	247
3. viii. 22	463	475	482	406
8. xi. 22	525	560	560	450
			Young red cock on 8. xi. 22:—430 g.	
8. i. 23	545	620	470	445
23. iii. 23	487	542	435	420
20. iv. 23	475	505	405	405

Other birds.

Day-old ducks, geese, turkeys, guinea-fowl, and pheasants have been used for ascertaining their B-vitamin requirements (see Part V, p. 794). They were all given a diet consisting of oatmeal and fish meal to which marmite and cod-liver oil were added. C-vitamin was not included.

Several losses amongst all the groups occurred, due to insufficient B-vitamin, but except in the case of turkeys, several birds were raised to maturity. One turkey was kept for 10 weeks. The other birds were kept for 12 to 16 weeks.

C-vitamin thus appears to be unnecessary for these birds.

Summary.

1. Pigeons have been kept for 15 months and have laid eggs and reared young on a diet without C-vitamin.

2. Chickens have been kept from the day old stage to maturity without C-vitamin. They have laid eggs and a few young chicks have been hatched from the eggs.

3. Ducks, geese, turkeys, guinea-fowl and pheasants have been reared from the day old stage for periods varying from 12 to 16 weeks without C-vitamin.

4. C-vitamin does not appear necessary in the food of domestic and farm birds.

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XCVII. THE REARING OF CHICKENS ON THE INTENSIVE SYSTEM. PART V. COMPARATIVE B-VITAMIN REQUIREMENTS OF PIGEONS, CHICKENS AND OTHER BIRDS.

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WITH THE ASSISTANCE OF

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(*Received September 4th, 1923.*)

It is very tempting to think that the food requirements of all species of birds are alike. Sugiura and Benedict [1923] from their experiments with pigeons have been led to generalise that very little A-vitamin is needed in *avian* nutrition. Emmett and Peacock [1923] do not agree with this generalisation and have found that chickens require more A-vitamin than pigeons. The experience of poultry keepers is also against any idea that all birds have the same requirements. It is held by them that turkeys and pheasants at any rate are much more difficult to rear than chickens. Our own data with reference to pigeons and chickens on diets of oatmeal [Part I, 1922] and rice [Part III, 1923] indicate that the B-vitamin requirements of chickens are greater than those of pigeons. It is possible that the difficulties of rearing turkeys and other farm birds may be partly connected with the B-vitamin needs. Some preliminary experiments have therefore been undertaken to study this question. They have indicated that B-vitamin is an important factor in the difference between the ease of raising of chickens and other birds. Further experiments cannot be made till the next season. The present incomplete data are now put forward as they may be of some little value to the poultry keeper.

Experimental.

Oatmeal and rice had been used as the cereal in the previous experiments with chicks and pigeons. As source of protein the chicks had generally been given dried skim milk and the pigeons fishmeal.

With oatmeal, pigeons had been kept for 15 months without any addition of marmite as source of extra B-vitamin. Chicks had an addition of 0·75 g. of marmite per 90 g. of oatmeal.

With rice, at the time these experiments were started, both chicks and pigeons had an addition of 3 g. marmite per 90 g. rice. Dried skim milk had probably considerably more B-vitamin in it than fishmeal.

No definite conclusion could be drawn from these data, but it was likely that the B-vitamin requirements of chickens were greater than those of pigeons.

The pigeons were already on a diet of oatmeal (90 g.) and fishmeal (5 g.). To avoid changing their diet, and as chickens had previously been kept on oatmeal the pigeon diet was chosen for the comparative experiments.

The mixture of oatmeal and fishmeal had the following composition:

Water	Salt	Ash	Fibre	Protein	Fat	Carbohydrate
7·9 %	0·2 %	2·7 %	0·9 %	14·5 %	8·2 %	65·8 %

with a protein to fat and carbohydrate ratio of 1 : 5·1.

The amount of ash was suitable, but the A-vitamin content was probably low. In the earlier experiments, no addition of cod-liver oil was made to supply A-vitamin, but later first 0·5 cc., then 1·0 cc., was added per 90 g. of oatmeal, in order to avoid complications on this account.

The experiments were not made in the order described but as follows: chicks, ducks, geese, ducks, guinea fowl, chicks, turkeys, pheasants.

Pigeons. The pigeons were kept on the same diet as described in Part IV. They were not able to rear their young without an addition of extra B-vitamin. Further evidence on this finding must await the next breeding season.

Chickens. Group X. It was not expected that chicks could be reared on oatmeal and fishmeal without the addition of marmite, but since the experiment had not been made it was necessary to establish the supposition, by allowing the birds to die if the food was not suitable.

The experiment was started on Apr. 7th, 1923, with ten day-old Light Sussex chicks.

One bird was unwell on arrival and died the next day. A second bird became ill on Apr. 9th, and died on the 8th day. All the birds showed ruffled feathers on the 12th day. A third bird was "off its legs" on the 17th day and died the following day. A fourth and fifth died on the 20th day. A sixth and seventh were ill on the 30th and 32nd days, and died on the 36th day. An eighth died on the 37th day. A ninth on the 39th day and the last on the 52nd day.

Post mortem examinations were made on the birds. In some cases nothing abnormal could be discovered. In others, the intestines were found full of undigested food. The chicks evidently suffered from constipation and died from absorption of toxic products formed by putrefaction in the gut. In two cases, a small swelling of the gut wall was found with a possible perforation. Chicks cannot thus be reared on a diet which suffices for adult pigeons.

Group XI. To prove that extra B-vitamin is needed by chicks on the oatmeal and fishmeal diet of pigeons, an addition of 1·5 g. of marmite per 90 g. of oatmeal was made. At the same time extra A-vitamin was included as cod-liver oil at the rate of 1 cc. per 90 g. of oatmeal.

The group consisted of 24 Black Leghorn day-old chicks and the experiment was started on June 1st and concluded on Aug. 17th, 1923 (11 weeks). One bird died on the second day. An unabsorbed yolk sac was found on post mortem examination. The remaining 23 birds were reared without any loss. Throughout the time of experiment the birds looked perfectly well and never showed signs of illness. The cockerels of the group began crowing in the seventh and eighth weeks. The best proof of their health is given by their steady rate of growth:

Average weekly weights.

	June 1	June 8	June 15	June 22	June 29	July 6
	g.	g.	g.	g.	g.	g.
	34·4	58·0	78	130	182	270
Increase	—	24	20	52	52	88
	July 13	July 20	July 27	Aug. 3	Aug. 10	Aug. 17
	g.	g.	g.	g.	g.	g.
	314	411	502	607	737	837
Increase	44	97	91	105	120	100

On the above diet the addition of 1·5 g. of marmite or 0·8 % of the food is sufficient for the health and growth of chicks. It is possible that 0·75 g. would be enough, but the more delicate birds would probably be lost.

Ducks. I. Aylesbury. The experiment was started on Apr. 12th, 1923, with four day-old Aylesbury ducklings.

During the first week there was an average gain in weight of 28 g.

One duck died during the second week; the others gained 21 g. Two other ducks died during the third week. The last duck died on the 30th day.

The pigeon diet was therefore not suitable.

II. White Runner. A group of 12 day-old White Runner ducklings was started on the oatmeal and fishmeal diet with an addition of 0·75 g. of marmite on Apr. 30th, 1923. Most of the ducks were shaky on their legs on the fourth day and seemed to have little appetite; they seemed better on the fifth day.

On the sixth day, five of them showed the typical symptoms of polyneuritis with the head bent back as is seen with pigeons. They were dosed with 2 cc. of 2 % marmite. Three died, but the other two were better; they died, however, on the next day.

0·75 g. marmite was obviously insufficient. It was raised to 2·25 g.

As no extra A-vitamin was present in the food, 0·5 cc. of cod-liver oil was included on the 10th day.

With the extra marmite the ducks flourished for the next two weeks. As it was desired to find the lowest amount of marmite necessary, the quantity was reduced on the 22nd day to 1·5 g. per 90 g. of oatmeal.

After this reduction another duck died on the 30th day; the gut was found full of food and perforated by a piece of undigested oatmeal.

During the sixth week, two ducks were noticed with a sort of contracted neck; one of them was better the next day; the other recovered after receiving several doses of 1 g. marmite dissolved in about 10 cc. of water.

As the ducks were getting ill on 1·5 g. additional marmite, the amount was obviously insufficient and it was raised to 2·25 g. on the 40th day. On the 47th day the cod-liver oil was raised to 1 cc.

After these additions all the ducks were kept in good health till the experiment was ended on 17th Aug. The duck which had suffered from the contracted neck and recovered after the doses of marmite always remained smaller than the others.

Six out of twelve ducks were reared with the addition of 2·25 g. of marmite, the other six died with a smaller quantity per 90 g. oatmeal.

The average weekly weights show that the final amount was sufficient:

	Apr. 30	May 7	May 14	May 21	May 28	June 4	June 11	June 18
	g.	g.	g.	g.	g.	g.	g.	g.
	36·3	56·8	127·7	265	411	690	817	1036
Increase	—	20·5	70·9	137	146	288	118	219

	June 25	July 2	July 9	July 16	July 23	July 30	Aug. 6	Aug. 13
	g.	g.	g.	g.	g.	g.	g.	g.
	1197	1305	1473	1454	1543	1629	1578	1603
Increase	161	108	168	−19	89	86	−51	25

The two occasions on which a decrease in weight was observed were during very hot weather, not much food being eaten.

Geese. This experiment was started with 13 day-old goslings on Apr. 26th, and concluded on Aug. 17th, 1923 (16 weeks).

It had been found that chicks and ducklings could not be reared on the oatmeal and fishmeal diet without additional B-vitamin. It was not likely that it would be suitable for day-old goslings, so 0·75 g. of marmite was included at the start.

The birds were rather unsteady on their feet on arrival, but looked better after a few days, though they were very quiet. They gained on the average 51 g. in weight in the first week.

Two died on the eighth day. No cause of death could be discovered on post mortem examination.

The marmite was therefore raised to 1·5 g. per 90 g. oatmeal.

Two more died on the ninth day. Since the others looked ill, they were given a dose of 4 cc. of 2 % marmite for the remainder of the second week. Six more however died. Post mortem examination showed the gut filled with food, largely undigested; in one case there was perforation of the intestine. Death was probably due to absorption of toxic products from the stagnated food in the gut.

At the beginning of the third week, 0·5 cc. of cod-liver oil was put into the food. It was increased to 1 cc. per 90 g. oatmeal at the seventh week to conform with the food of the other kinds of birds.

The three remaining goslings survived the fourth week though their condition was not satisfactory. One died at the beginning of the fifth week. Marmite was then raised to 2·25 g. per 90 g. oatmeal.

After this addition the last two geese were kept till Aug. 17th without any illness. Their final weights averaged 5835 g.

2·25 g. marmite is thus needed by geese on the oatmeal and fishmeal diet.

Guinea Fowl. Five day-old and six rather older guinea fowl were used for this experiment. It was started on May 18th and concluded on Aug. 17th (13 weeks).

From the first day an addition of 1·5 g. marmite and 0·5 cc. of cod-liver oil was made. To conform with the other groups the cod-liver oil was increased to 1 cc. per 90 g. of oatmeal at the beginning of the fourth week. Two birds died during the first week; one of them had its caeca full of food blocking the passage through the gut. The others at this period had very little appetite. On this account the marmite was raised to 2·25 g. per 90 g. oatmeal (May 25).

A third died during the second week, and a fourth during the third.

No further loss occurred. The remaining seven were kept without any signs of illness. Their feathering was perhaps not as good as is seen normally. The growth of the birds is seen by their average weekly weights:

May 18	34 g.	June 1	92 g.	July 6	463 g.	Aug. 3	830 g.
,, 25	57	,, 8	156	,, 13	551	,, 10	966
		,, 15	212	,, 20	619	,, 17	1028
		,, 22	295	,, 27	724		
		,, 29	359				

Again, 2·25 g. is the required addition of marmite to the diet.

Turkeys. Ten day-old turkey poults were started on the diet with 1·5 g. marmite and 1 cc. of cod-liver oil on May 26th. One bird was kept till Aug. 5th, 1923. The birds did not like the diet and ate very little. At the end of the first week six had died. The others only gained an average of 6 g. Marmite was at once raised to 2·25 g. per 90 g. of oatmeal.

With this increase, the remaining four gained 15 g. during the second week. An unfortunate accident then occurred in which three of them were lost by fire in the foster mother. The last bird grew steadily each week, but in the tenth week it met with some accident in which its legs were damaged and it was chloroformed.

It appears thus that 2·25 g. of marmite is just sufficient for rearing turkeys on the oatmeal and fishmeal diet.

The bird weighed 560 g. at the end of the tenth week.

Pheasants. These birds were most kindly supplied for the experiment by Mr J. Q. Rowett. The experiment was started on June 4th and terminated on Aug. 27th, 1923. 1·5 g. of marmite and 1 cc. cod-liver oil were added to the food mixture at the start.

One bird died during the second week; no definite symptoms could be found on post mortem examination. Another broke its leg and was chloroformed. During the third week three died. These were found to have enlarged caeca causing stoppage in the gut.

The marmite was raised to 2·25 g. after these losses. Except for one more loss in the fifth week, the others were reared for 12 weeks. Unfortunately

one of them damaged its leg and had to be chloroformed. Though they lived and grew quite well, it was very noticeable that the food was not entirely suitable; their sizes were very unequal and their feathering was very poor. The larger birds were continually pecking at new feathers which formed on the smaller ones.

Their rate of growth was slow as seen from their weekly weights:

June	4	18·8 g.	July	2	72 g.	Aug.	6	240 g.
,,	11	25	,,	9	96	,,	13	295
,,	18	35	,,	16	132	,,	20	356
,,	25	49	,,	23	174	,,	27	422
			,,	30	200			

General Remarks.

Except adult pigeons and chicks, all the birds have needed an addition of 2·25 g. of marmite per 90 g. of oatmeal for their successful rearing. Chicks required 1·5 g.

This quantity is the minimum. Better results would undoubtedly have been secured with an addition of 3 g. per 90 g. oatmeal. 1 cc. of cod-liver oil per 90 g. oatmeal is sufficient.

The diet was specially chosen with only 5 g. of fishmeal. An increase to 10 g. would be likely to give very satisfactory growth. As pointed out in Part III, a change of cereal might not lead to a successful result since the quantity of B-vitamin in cereals is not yet known.

A diet upon which adult pigeons can exist is certainly inadequate for all other farm birds. It is curious that chickens have a smaller B-vitamin requirement than the others. It is possible that they are able to digest oatmeal better and thus obtain the whole of the B-vitamin present in it, whereas some of this B-vitamin is not assimilated by the other birds. The difference is not great.

Summary.

Pigeons on 90 g. oatmeal and 5 g. fishmeal do not require any extra B-vitamin.

Chickens on the same diet need an addition of 1·5 g. marmite per 90 g. of oatmeal.

Ducks, geese, guinea fowl, turkeys, pheasants, need an addition of 2·25 g. of marmite to supply extra B-vitamin.

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XCVIII. UREASE. PART II. THE MECHANISM OF THE ZYMOLYSIS OF UREA.

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(*Received September 5th, 1923.*)

In a previous communication [Fearon, 1923] an account was given of the detection and estimation of cyanic acid in solutions of urea undergoing zymolysis, and it was suggested that cyanic acid and ammonia are the intermediate stages in the enzymic decomposition of urea:

$$\underset{\text{Urea}}{CON_2H_4} \begin{cases} \longrightarrow NH_3 \\ \searrow \underset{\text{Cyanic acid}}{HNCO} \end{cases} \xrightarrow{+H_2O} NH_3 + CO_2$$

Since this paper was submitted for publication, a paper has appeared on the mechanism of urease by Mack and Villars [1923]. In this paper the authors agree with the present writer in accepting the Werner formula for urea. They have detected and estimated cyanate in solutions of urea undergoing zymolysis, and also have shown that the cyanate rises to a maximum and falls again towards the end of the zymolysis. Their maximum values expressed as percentage cyanic acid agree closely with several maximum values obtained by the present writer (0·003 %).

On the other hand, they arrive at the conclusion, by a process of elimination, that the transformation

urea → ammonium carbamate

is the stage catalysed by the enzyme, and they do not appear to consider cyanic acid as a possible intermediate product in the zymolysis.

This conclusion is based on indirect evidence; carbamic acid or carbamates have not been shown to be present in the urea/urease system. The work of Yamasaki [1918] is inconclusive in this respect, since the experimental method he adopts will not discriminate between carbamates and cyanates.

The method employed by Mack and Villars in estimating cyanic acid consists in precipitating the samples with excess of silver nitrate, washing the precipitate free from ammonia, digesting with dilute nitric acid to convert the cyanate into ammonium nitrate, and estimating the ammonia so formed by nesslerisation.

When silver nitrate is added in excess to a mixture of ammonium carbonate, carbamate, and cyanate in solution, a precipitate is thrown down consisting of the silver salts of the three acids. If the solution be now set to p_H 5 by means of dilute nitric acid the carbonate and carbamate dissolve leaving the silver cyanate. This is the basis of the method adopted by the present writer in estimating cyanic acid [1923].

The method of Mack and Villars is open to the objection that any carbamate present would be included in their result as cyanate; this defect can be overcome by setting the solutions to a suitable p_H before filtration. Thus modified, their method is much more convenient than that formerly adopted by the present writer, especially now that a preparation of urease containing a minimum amount of protein is available (*urease Dunning*).

To determine the exact significance of the Mack and Villars results, and to obtain information as to the part played by carbamate in the urea/urease system, the cyanate and carbamate in solutions of urea undergoing zymolysis were determined simultaneously.

Estimation of the Carbamate and Cyanate in the Urea/Urease System.

Solutions of pure urea and urease (*Dunning*) of such proportions as to produce 0·1 *M* urea and 0·1 % urease were mixed and incubated at 20°. At definite intervals duplicate samples of 50 cc. were withdrawn and treated with excess of *N*/1 $AgNO_3$. One sample was filtered at once, this gave the carbamate and cyanate residue. The other sample was set to p_H 5 by means of 0·6 *N* HNO_3, using methyl-red and a comparator, and then filtered, this gave the cyanate residue only. The residues were washed until free from ammonia, and then were digested on a water-bath with 20 cc. of 2 % HNO_3. After cooling, they were treated with 15 cc. of saturated K_2CO_3, diluted to 50 cc. and the ammonia determined by aspiration into 100 cc. of *N*/500 HCl, and subsequent nesslerisation. The results are expressed as nitrogen derived from both carbamate and cyanate and from cyanate alone in the 50 cc. samples. The difference between these values represents carbamate alone. By titration of samples from time to time with 0·6 *N* HNO_3, using methyl-red, the progress of the zymolysis was roughly determined.

Table I.

Time in minutes	Carbamate and cyanate N in 50 cc. × 10^4 g.	Cyanate N in 50 cc. × 10^4 g.	Cyanic acid %	Urea % decomposed
0	0	0	0	0
30	1·1	1·0	0·0006	18·6
60	2·3	2·5	0·0015	—
130	5·2	5·0	0·0031	52
240	2·0	2·3	0·0014	91
1320	0·1	?	0	99·9

Substrate 0·1 *M urea.* *Temperature* 20°.
Enzyme 0·1 % *urease Dunning.*

From these results it will be seen that the cyanate N and the cyanate N plus carbamate N agree; and consequently there is no indication of the presence of carbamate at any stage during the zymolysis of urea. The results of Mack and Villars may be taken as representing cyanate, as will be seen from a comparison of their figures with the above table.

Table II. (*From the data of Mack and Villars* [1923])

Time in minutes	N in 50 cc. $\times 10^4$ g.	Cyanic acid %
5	2·33	0·0014
10	2·75	0·0016
20	3·46	0·0021
80	5·59	0·0034
165	3·99	0·0024

Substrate 0·1 *M urea.* *Temperature* 25°.
Enzyme 0·1 % *urease.*

Mack and Villars conclude that the cyanate in the urea/urease system is due to the spontaneous dissociation of the urea, and is uninfluenced by the presence of the enzyme.

That this is not so was demonstrated by a control solution of urea alone, which showed no indication of cyanate formation during the time of the experiment, nor for several days unless it had become infected.

The Urea-Ammonium Cyanate Equilibrium.

Urea in aqueous solution when kept *sterile* appears to be stable at ordinary temperatures for an indefinite period. Werner [1918] has found $N/2$ solutions of urea in toluene water free from cyanate or carbonate after nine months. On the other hand, a similar solution of urea in plain water showed signs of decomposition after 14 days at room temperature. A 5 % solution of urea kept by the author under ordinary laboratory conditions for one year showed on analysis a conversion of 0·2 % of the urea into ammonium carbonate. A mould with urealytic properties was found to have developed in the liquid. The solution contained no cyanate; the activities of the mould had presumably been inhibited by the increasing alkalinity of the solution, and the cyanate had been hydrolysed to carbonate.

Werner explains the stability of urea in sterile solutions below 40° as being due to the fact that it is not dissociated into ammonia and cyanic acid under such conditions. If, however, these solutions are heated to about 60° dissociation occurs with production of cyanic acid and ammonia. This dissociation has been very fully investigated by Walker and Hambly [1895], who have shown that, after one hour at 100°, 4 % of a $N/10$ urea solution had come into an equilibrium with ammonium cyanate produced by the transformation of the urea.

The equilibrium concentration for 39° found by incubating $N/10$ solutions containing 95 % urea and 5 % ammonium cyanate, is open to several experimental objections. Part of the cyanate is converted into urea and part is hydrolysed to ammonium carbonate.

The urea-ammonium cyanate equilibrium is not stable as the cyanate is gradually hydrolysed to ammonium carbonate until all the urea is removed from solution by this double process of dissociation and hydrolysis.

This change is slow if the ammonia be prevented from leaving the system. Fawsitt [1902] found that 450 hours were required to bring about the de-

composition of 98·9 % of the urea originally present in a semi-normal solution kept at 99° in a sealed tube.

The Formation of Ammonium Carbamate from Urea.

The direct formation of ammonium carbamate by the hydration of urea has not been demonstrated experimentally. Its possible occurrence is open to doubt. The various syntheses of urea from ammonium carbonate and from ammonium carbamate have been shown by Werner to involve an intermediate formation of cyanic acid from the dehydration of carbamic acid. It is hoped to discuss the very interesting subject of the enzyme synthesis of urea from ammonium carbonate and carbamate claimed by several investigators [Barendrecht, 1919; Mack and Villars, 1923; Kay, 1923] in a subsequent paper.

Cyanic Acid as the Intermediate Stage in the Urea/Urease System.

Before a compound can be considered as a possible intermediate stage in a catalysed reaction it must be shown to conform to the requirements of Ostwald's law of successive reactions [1902]. The intermediate reaction must take place more rapidly than the direct reaction under the same conditions. The concentration of such a compound will depend on its stability. As Hopkins has observed: "the degree to which a substance accumulates is itself no measure of its metabolic importance, no proof as to whether it is on some main-line of change, or a stage in a quantitatively unimportant chemical by-path" [Bayliss, 1917]. Cyanic acid in aqueous solution is unstable, and the degree to which it accumulates will depend on the difference between the rate of formation and the rate of removal. At the start of the reaction the cyanic acid gradually reaches a maximal concentration depending on the temperature and concentration of the enzyme, but independent of the concentration of substrate, provided that there is excess of substrate.

Working with low concentrations of enzyme preparation (0·1 to 0·2 %) and substrate (0·1 to 0·5 *M* urea) at temperatures of 5 to 20° the maximum concentration of cyanic acid is 3–4 mg. %. The figures obtained by the method of Mack and Villars, adopted in the present paper, are probably more accurate than those obtained by the use of collodion sacs described in the previous paper [Fearon, 1923], owing to the diffusion being retarded by the sac.

Towards the end of the zymolysis the rate of formation of cyanic acid begins to decrease owing to decrease of substrate and accumulation of ammonia in the system, which retards the hydrolysis of cyanic acid, as well as the dissociation of the substrate. Consequently, the removal of cyanic acid decreases in rate also towards the close of the reaction.

The Hydrolysis of Cyanic Acid.

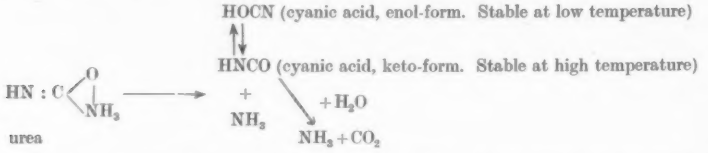
In the liquid state below zero, and in aqueous solutions, cyanic acid is an equilibrium mixture of the two forms:

HO . CN (Enol-form, stable only at low temperature) $\rightleftharpoons$ HN . CO (Keto-form, or iso-*cyanic acid*, stable only at high temperature)

Liquid cyanic acid, below zero, contains about 60 % HOCN and 40 % HNCO. Rise of temperature leads to increase in HNCO, and fall of temperature increases the HOCN [Werner and Fearon, 1920]. Union of enol-cyanic acid with ammonia produces ammonium cyanate.

Union of keto-acid with ammonia produces urea.

When urea in solution undergoes dissociation, ammonia and cyanic acid in the keto-form are produced. Keto-cyanic acid is less stable at low temperatures than the enol-form of the acid, accordingly, some of it is transformed into enol-cyanic acid and some of it is hydrolysed to ammonia and carbon dioxide. The result may be represented as follows:



Owing to the existence of these two forms, the hydrolysis of cyanic acid in aqueous solution is complicated, and involves three distinct primary changes [Werner and Fearon, 1920]:

(1) $(HOCN \rightleftarrows HNCO) + H_2O = CO_2 + NH_3$.

(2) $HOCN + NH_3 = NH_4 . OCN$ (ammonium cyanate).

(3) $HN : CO + NH_3 = HN . CONH_3$ (urea).

At the outset of the reaction the keto-cyanic acid is hydrolysed with the production of CO_2 and NH_3. In 15 minutes 15 % of a $N/4$ solution of cyanic acid at 0° was converted into CO_2 and NH_3. As the ammonia gradually begins to accumulate in the solution it unites with the keto-acid to form urea, and the hydrolytic process gradually slows down.

In the presence of urease the hydrolysis of cyanic acid is considerably modified. First, since the urea molecule is decomposed at a comparatively low temperature (0° to 55°) most of the cyanic acid is liberated into solution in its less stable form and is more readily hydrolysed than if liberated from a normal cyanate. Then, urease is able to combine with ammonia, reducing its concentration in the liquid phase of the system. Also, the urea formed synthetically during the late stages of the hydrolysis of the cyanic acid is in turn broken down by the enzyme.

All these factors will increase the amount of free keto-cyanic acid in the solution, which is the form in which the acid is most readily attacked by water.

The Mechanism of Urease Action.

As the present paper is chiefly concerned with the chemistry of the zymolysis of urea the interpretation of the data obtained from the study of the rates of zymolysis under varying conditions will not be discussed. The

most comprehensive account to date will be found in the work of Lovgren [1921], which includes an exhaustive bibliography of 212 references. The problem at issue can, however, be restated in more precise terms in view of the detection of cyanic acid in the urea/urease system. The urealytic property of the enzyme is due to its power of dissociating urea in solutions at ordinary temperatures, from 0° to 60° (above which the enzyme is rapidly destroyed).

The Adsorption Stage in the Urea/Urease System.

With the exception of Barendrecht [1919] and Yamasaki [1918], there is general agreement amongst the investigators of the kinetics of the urea/urease system that the first stage in the zymolysis is a union between the substrate and the enzyme. The precise nature of this union has not yet been determined. Armstrong, Benjamin and Horton [1913], Bayliss [1915, 1918] have come to the conclusion that urease acts by surface condensation or adsorption. Van Slyke and Cullen [1914] consider that the change is due to a chemical combination of enzyme and substrate, followed by a decomposition of the intermediate product according to the laws of chemical reactions deduced from mass action.

In support of the adsorption theory, Bayliss [1915] has described some simple and convincing experiments in which he shows that urease is able to act in a medium in which it is quite insoluble. Working with solutions of 4 % urea in 80 % to 89·3 % alcohol he found that urease was able to decompose from 7·8 % to 1·8 % of the substrate although the enzyme was quite insoluble in the medium.

The Action of Urease in Absolute Alcohol.

The determination of the degree of adsorption of urea by urease was attempted by extending the experiments of Bayliss to solutions of urea in absolute alcohol.

A preparation of soy-urease, freed from fat by ether extraction in a Soxhlet extractor, was extracted at 40° with absolute alcohol until the filtrate no longer gave an opalescence on dilution with water and addition of silver nitrate. 50 g. of this preparation, which was very active, were added to 50 cc. of a saturated solution of pure urea in absolute alcohol at 18°. The mixture was incubated in a closed flask at 18°, and frequently shaken. A similar flask containing a saturated alcoholic solution of urea was kept under the same conditions as a control. The urea in each solution was determined from time to time by drawing off 2 cc. samples and determining the urea gravimetrically after evaporation of the alcohol on a water-bath. Equilibrium was reached after ten days.

Table III.

	%	
Urea in control solution	4·290	
„ in presence of enzyme	3·475	
„ removed from solution	0·815	
„ adsorption	19·0	of urea present

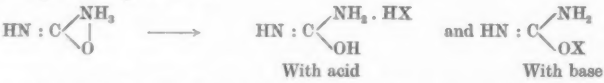
On filtering off the enzyme, the filtrate was found to contain no ammonia, but on dilution with water and after addition of silver nitrate gave a white precipitate soluble in dilute nitric acid, and responding to the test for cyanate by hydrolysis and nesslerisation, as described in Part I of the present communication [1923]. This was not observed in the control solution of urea, or in suspensions of the enzyme in absolute alcohol. The residue from the experiment was still active, and appeared unchanged.

This experiment supports the theory that adsorption is the preliminary stage in the zymolysis of urea. It also indicates that adsorption alone is inadequate to explain the change. Only a very small fraction of the adsorbed urea can have been dissociated.

It may be possible to find an explanation in terms of general adsorption for cases of enzymes which accelerate reactions, such as the hydrolysis of esters, where increase of concentration of the reactants in the surface layer will bring about increase of reaction-rate in accordance with the law of mass action. This subject has been recently reviewed by Bayliss [1919]. But in the case of enzymes such as urease, which initiate, or appear to initiate, reactions, general adsorption is not sufficient to explain the zymolysis, since a saturated solution of urea is quite as stable as a weak solution, in the absence of infection. Increase of concentration in the surface layer is by itself insufficient to bring about decomposition.

Selective Adsorption in the Urea/Urease System.

The adsorbent properties of urease, apart from its urealytic power, do not appear to have been investigated hitherto. Urea in neutral solution is present as a closed-ring compound. In this form it is not attacked by nitrous acid and does not give a condensation derivative with xanthhydrol [Werner, 1923]. The action of acids or alkalies is to open up the ring and form salts with the amphoteric *free* urea:



When the neutral urea molecule undergoes dissociation it gives rise to an alkaline component, ammonia, and an acid component, keto-cyanic acid. Acids or alkalies accelerate the decomposition of urea in aqueous solutions above the dissociation point by combining with either the alkaline or the acid component.

The behaviour of urease towards ammonia and cyanic acid was investigated in order to see if, in addition to its property of adsorbing urea, it showed any evidence of selective adsorption, or greater preference for one constituent of the system. Preliminary observations were made on the nature of the electric charge borne by urease in colloidal suspension.

Solutions of soy-urease, decanted from 5 % suspensions, and dialysed for a couple of hours against distilled water to remove excess of electrolytes,

were subject to a potential difference of 100 volts in a conductivity tube (Hardy's modification of Whetham's apparatus), giving a fall in potential gradient of 1 volt per cc.

The colloid showed an electro-negative charge. After 20 minutes at 15° the particles had accumulated in the region of the anode.

Control experiments with urease suspensions boiled for 10 minutes showed an almost complete disappearance of charge.

In the absence of more precise information as to the nature of the enzyme found in the urease preparations conclusions from experiments on impure preparations must be tentative. When an active preparation of urease free from foreign protein is available the work will be repeated.

The behaviour of soy-urease towards ammonia was then investigated. If the ammonia group of the neutral urea is adsorbed by the electro-negatively charged surface leaving the unstable cyanic acid in the outer zone of the Helmholtz "double layer," the rapid decomposition of the substrate should continue until the surface is saturated with adsorbed ammonia.

It is well known that ammonia retards the action of urease, as first shown by Armstrong and Horton [1912], but the fact that urease is able to adsorb ammonia has not been previously demonstrated.

The Adsorption of Ammonia by Urease.

Samples of soy-urease freed from fat were shaken up with solutions of ammonium sulphate of varying strength. After standing for 24 hours at room temperature, the mixtures were centrifuged and the ammonia in the supernatant liquid determined by the colorimeter.

The mixtures consisted of 2 g. soy-urease and 50 cc. standard ammonium sulphate (Folin). The average temperature was 18°.

Table IV.

	Before adsorption. Ammonia N in mg. %	After adsorption. Ammonia N in mg. %	Percentage adsorption
A.	28	16·6	40·8
B.	14	6·5	53·5
C.	7	0·0	100

A control solution of urease (4 %) in water alone did not liberate any ammonia after 24 hours.

The supernatant liquid from a 4 % solution of soy-urease in water alone, after standing for 24 hours before being centrifugalised, contained no ammonia.

For the purposes of comparison, the ammonia-adsorbing property of a sample of permutit was determined under the same conditions.

The mixture consisted of 2 g. of washed permutit and 50 cc. ammonium sulphate solution. Time, 24 hours. Temperature 18°.

Before adsorption. Ammonia N in mg. %	After adsorption. Ammonia N in mg. %	Percentage adsorption
28	6·6	76·4

Permutit does not adsorb urea in aqueous solution.

Urease is not destroyed by the action of ammonia or ammonium salts at ordinary temperatures. Suspensions of soy-urease in $N/10$ $NH_4.OH$ after three months at 12° to 14° regained urealytic activity on washing with 2% acetic acid and with water. Even solutions of urease incubated with excess of 10 N ammonium carbonate and carbamate for 10 hours at 55°, in accordance with the method of Mack and Villars [1923], for showing the synthesis of urea, on removal of the ammonia were active towards urea, although a considerable amount of the enzyme material had been destroyed by the action of the strong alkali.

The action of urease on cyanic acid is difficult to investigate, since it is not possible to obtain the unstable form of the acid in solution apart from interfering substances. Mack and Villars have studied the action of urease on 0·1 M solutions of ammonium cyanate (made by mixing equal parts of 0·1 M solutions of NH_4Cl and KCNO) at 25°.

They find that urea is formed more readily from such solutions in the absence of urease than in its presence, which is as might be expected, because the concentration of ammonia in their experiments is not sufficient to inhibit completely the action of the enzyme.

Urease does not increase the rate of formation of *ammonium cyanate* from urea, and consequently need not necessarily catalyse the reverse reaction. The action of urease is to liberate the unstable form of cyanic acid from urea; this is rapidly hydrolysed until the progress of the reaction has resulted in the accumulation of ammonia sufficient both to retard the enzyme by forming an adsorption compound to the exclusion of the substrate and to retard the hydrolysis of the keto-cyanic acid. Ammonium cyanate can only appear at a late stage in the urea/urease system.

The Significance of Ammonia Adsorption in the Urea/Urease System.

Ammonia adsorption may in itself be sufficient to account for the dissociation of adsorbed urea, but it must be remembered that several other factors are concerned. The concentration of adsorbed substrate is difficult to determine, and until it is known, the ammonia concentration on the enzyme surface can only be inferred from kinetic data.

Urea adsorption by urease is a rapid process in aqueous solution, as was found by addition of soy-urease to solutions of urea just above freezing-point, and rapid separation again of the enzyme by alcoholic precipitation and centrifugalisation. The residue was then taken up with water, and incubated. The adsorbed urea underwent decomposition, thus affording a rough method of determining the extent and rate of adsorption.

Substrate, 10 cc. of 2% urea. Temperature 0°.

Enzyme, 0·1 g. urease *Dunning*, in fine powder.

The mixture was shaken for 30 seconds, and then treated with 80 cc. absolute alcohol, which completely precipitated the enzyme. The mixture

was then rapidly separated by centrifugalisation, and the residue was incubated at 36°.

Ammonia formed = 19 mg. urea, or 9·5 % of substrate present.

By an extension of this method the rate of adsorption might be determined. The conclusions from qualitative experiments on the mechanism of enzyme action must be restricted owing to lack of knowledge as to the degree of purity of the various preparations, and the necessity for distinguishing between accidental and essential properties of the ferment.

Until urease is obtainable in a form of biochemical purity, corresponding to the *amylase* prepared by Sherman, an extension of the work outlined in the present paper does not appear to be profitable.

In addition to the effect of selective adsorption of ammonia it must be noted that adsorbed urea is probably subjected to a considerable mechanical pressure in the surface layer.

Williams [1920], working with charcoal, has calculated the surface pressure to be of the order of 50,000 atmospheres. Many of the instances of irreversible adsorption may be ascribed to compression effects, such as the coagulation of adsorbed proteins at ordinary temperatures, a change which in the absence of the adsorbent requires pressures of 17,000 to 20,000 atmospheres [Bridgman 1914].

The dissociation of urea gives rise to the very compressible base ammonia, hence it is to be expected, although it has not yet been demonstrated, that high pressures alone should bring about the dissociation of urea. Lewis and Burrows [1912] have found that the decomposition of urea in solution by heat is always accompanied by a diminution in volume. It has also been found that in the Haber ammonia process increase of pressure displaces the equilibrium so that more is formed of the component occupying the lesser volume, namely ammonia [Haber, 1914].

A Theory of the Mode of Action of Urease.

From qualitative data it is possible to construct a theory of the mode of action of urease, which will explain, amongst other things, the specific activity of the enzyme.

Urea is adsorbed from solution by urease and then dissociated into ammonia and cyanic acid in its unstable form. The ammonia is adsorbed by the enzyme, the cyanic acid remains in solution.

In aqueous solutions the cyanic acid is rapidly hydrolysed into ammonia and carbonic acid. The resulting ammonium acid carbonate is able to combine with another molecule of ammonia, and so will tend to reduce the concentration of adsorbed ammonia, thus freeing the enzyme.

For this reason, acids in low concentration accelerate the reaction by combining with the ammonia. Alkalies in low concentration retard the zymolysis by neutralising the carbonic acid, by fixing the cyanic acid as a much more stable cyanate, and also by being adsorbed themselves.

Carbonic acid being a weak acid, and its salts with ammonia being largely dissociated, a stage is reached in the urea/urease system when the enzyme is approaching saturation with ammonia, and the reaction rate steadily falls, depending on the diffusion of the end-products into the solvent. If at this stage the H-ion concentration of the solution be increased to its optimum range (p_H 6·8–7·0) by buffers or by aeration with carbon dioxide the reaction rate returns to its maximum, provided the substrate be in excess. Under such conditions the rate of the zymolysis approximates to linear [Armstrong and Horton, 1912]. Here, what is being measured is the rate of hydrolysis of free cyanic acid at an optimum H-ion concentration.

Acids in high concentration stop the zymolysis of urea completely; the action is probably due to the precipitation of the enzyme, either directly or associated with the globulin of the urease preparation.

In higher concentrations of acids some of the urease preparation goes into solution once more, but has no action on urea. This may be due to a reversal of the electric charge, but until the purification of urease has been accomplished the subject cannot be thoroughly investigated.

An additional, but probably less important, effect of acids is their action on the substrate itself, opening up the cyclic form of urea to produce the salt form which is not dissociated into cyanic acid and ammonia.

Reversibility of Action of Urease.

Since ammonia and carbon dioxide are not the primary products of the action of urease on urea, its synthesis from these substances is not an inevitable consequence of the general theory of enzyme action. Mack and Villars [1923] and Kay [1923] have undoubtedly obtained urea from the action of strong solutions of ammonium carbamate and carbonate on urease. However, it has not yet been demonstrated that this urea arises from an enzyme dehydration of the carbamate, and it appears unlikely, since Werner has shown that the classical Basarov synthesis [1868] of urea by the action of heat on ammonium carbamate involves the intermediate formation of cyanic acid, as first suggested by Mixter [1882].

The results of an extended investigation following the lines of Mack and Villars will be described in a subsequent communication.

Summary of the Dissociation Theory of Urease Action.

(1) At the beginning of the reaction the urea/urease system consists of the non-electrolyte urea in true solution, and the enzyme urease in suspension as a negatively-charged colloid:

(2) *Stage of Adsorption* (occurs in alcoholic and aqueous solutions). Urea is withdrawn from solution, and concentrated on the enzyme surface:

(3) *Stage of Dissociation.* Adsorbed urea is dissociated into keto-cyanic acid and ammonia, which is held by the enzyme:

$$HN : CO + NH_3 \text{ (urease)}$$

(4) *Stage of Hydrolysis* (occurs in aqueous solutions only). The keto-cyanic acid is hydrolysed by the solvent, with the formation of ammonia and carbonic acid:

$$HN : CO + 2H_2O = NH_3 + H_2CO_3 \rightarrow NH_4 . H . CO_3$$

(5) The carbonic acid and acid carbonate combine with some of the adsorbed ammonia, and liberate the enzyme:

$$NH_4 . H . CO_3 + NH_3 \text{ (urease)} \rightarrow (NH_4)_2CO_3 + \text{(urease)}$$

(6) The liberated urease combines with more urea, and so the cycle continues, until brought to rest by exhaustion of substrate or by accumulation of ammonia in sufficient concentration to inhibit stage (5). Stage (5), the breakdown of the adsorption compound, is the true characteristic of the urea/urease system (and probably all catalytic actions in heterogeneous systems). In the absence of stage (5) there can be no continuous zymolysis; the chemical changes would be linear, not cyclical.

Conclusions.

1. No evidence has been obtained of the occurrence of carbamic acid in the zymolysis of urea. Cyanic acid and ammonia appear to be the intermediate products of the action of urease on urea. Urease acts by dissociating its substrate.

2. Soy-urease is able to adsorb urea from alcoholic solution.

3. Soy-urease in aqueous solution carries an electro-negative charge, and is able to adsorb ammonia from aqueous solution.

4. A theory of the mechanism of urease is presented in outline. Urease combines with urea by adsorption. The adsorbed urea is dissociated into ammonia, which combines with the enzyme, and cyanic acid, which is hydrolysed by the solvent. The enzyme is then liberated from the ammonia compound.

5. It is suggested that urea dissociation is brought about by selective adsorption and also possibly by the pressure in the surface layer.

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XCIX. THE INFLUENCE OF SURFACE TENSION ON THE GROWTH OF BACTERIA.

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A report to the Medical Research Council.

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One of the factors which may be anticipated to affect the growth and metabolism of bacteria is surface tension.

The relation of this physical factor to other biological problems such as muscle action or the deposition of salts in plant cells [Macallum, 1911] has been the subject of much study; the relation to bacterial growth has received little attention.

If, however, one considers the results obtained with surface films by J. J. Thomson [1888] and others it is evident that the increased concentration of substances, such as peptone, at the air/liquid interface ought to have a pronounced effect on those organisms growing at the surface. This should especially be the case with micro-organisms like *C. diphtheriae* where experience has shown that efficient toxin production is associated with good pellicle formation.

In a study of certain factors affecting toxin production my attention was drawn to this aspect of the subject by the statements of practically every bacteriologist who has worked in this field as to the necessity of removing all but inevitable traces of fat from the medium before it is used. The fact that pellicle is also essential would appear to be connected with the particular conditions of the air/liquid interface.

In a paper published in 1919 Larson, Cantwell and Hartzell [1919] made some very interesting statements regarding the effect of lowering the surface tension of media on the growth and habit of bacteria. The protocols of their experiments are unfortunately lacking in details of the methods used for determining surface tension. On lowering the surface tension of their media they observed that anaerobic bacteria grew in the presence of air, that organisms, usually forming pellicles, grew throughout the liquid and did not form a film and that spore-bearing organisms no longer produced spores. Evidently the unusual environment of the bacteria completely changed their habit of growth.

As my interest was in the behaviour of organisms whose pellicle forming properties are important, I devoted some attention to a study of the effects of lowered surface tension on the growth of *C. diphtheriae* and extended these observations to other organisms.

Certain general observations may be made here regarding surface tension, although the subject is discussed among others by Freundlich [1922] and Bayliss [1923].

In the first place no standard method has as yet been arrived at for the determination of its value. In most instances in biological research the stalagmometer has been used and has proved to be a satisfactory instrument if employed in a strictly comparative way. It is in the comparison of the results of one worker with those of another that difficulties arise. This was seen in the meiostagmin reaction of Ascoli and Izar [1910], which is possibly of real biological significance and yet has been found so difficult to repeat by other workers that it has not had general acceptance.

The stalagmometer represents the transition from a purely dynamic method such as the jet method of Lord Rayleigh and the static method of either the capillary tube or the measurement of pull of a solid being torn from the surface of a liquid. Both static and dynamic methods give practically concordant results when pure non-associating liquids are dealt with. It is only when we come to deal with liquids containing substances depressing or elevating surface tension that we find difficulties.

With the drop weight method, the longer the time taken to form the drop, the lower or higher the surface tension value obtained, until a number is reached which is close to that obtained by the static method. That this is so will be seen in some of the present results. The earlier values for bacteriological media obtained with the stalagmometer and only moderately slow dropping are very close to those given by Larson and his co-workers. When the number of drops was decreased and the time taken for each individual drop to fall very much prolonged, values were obtained which were about 10 dynes per cm. lower than in the first method, and these were very close to those obtained by the static ring method to be described later.

Larson, Cantwell and Hartzell [1919] discuss some of the possible causes of the change in the behaviour of bacteria through lowering the surface tension. They point out that the conditions for bacterial nutrition are very different at the surface from those in the interior owing to the great increase in the concentration of nutrient substances such as peptone in the surface layer. This must be correct, for Metcalf [1905] in investigating the behaviour of thin films of peptone solution showed that these consisted of a layer of almost undiluted peptone. We also know that other thin films of protein solutions such as the froth of egg-albumin consist almost entirely of the solid component.

The conclusions of Larson cannot altogether be accepted without reserve. The bacteria/water interface must also present an enrichment atmosphere and before it can be assumed that the air/water interface is a situation of

increased concentration of nutritive substances for the cells we ought to be informed regarding the system chitin/water or hemicellulose/water concentration differentiated with the air/water system before we can generalise respecting situation of increased or decreased nutritional environment.

For lack of more precise information the terms chitin and hemicellulose are here used to designate the composition of the bacterial envelope. It seems more than probable that the composition varies, and it may be this very reason which determines that some bacteria in their search for the most suitable surroundings seek the surface of the liquid while others are attracted to the interior of the medium.

There is also an important effect which is not mentioned by Larson and his colleagues. It is known that in the contest for the surface layer those substances which are more powerful in decreasing the surface tension tend to remove those which are less efficient. This may be the double cause of the deleterious action of small quantities of fats when these are not removed from the medium. They not only lower the surface tension but in so doing expel the nutrient substances of less surface tension-depressing power from the surface of the liquid. Thus the surface layer is no longer as rich in nutrient substances but has a high concentration of substances such as soaps which may be directly toxic to those bacteria existing near the interface. In speaking of the interface not only is the air/water junction implied, but also the bacterial/aqueous interface.

Experimental.

The present experiments were designed to enlarge the observations of Larson, Cantwell and Hartzell, and if possible throw some light on the relation between changes in surface tension and the pellicle formation of *C. diphtheriae*. As Larson and his colleagues had recommended the use of sodium ricinoleate for changing the surface tension of the medium it was decided to employ it. The sample used was made from castor oil by following their directions. A 5 % solution of the soap in water was used as a stock solution.

In the first experiments a stalagmometer of the Traube [1887] type was used, dropping at the rate of 15 drops per minute. The drops fell directly into free air and were counted. As a preliminary, certain combinations of nutrient broth made from English and imported beef combined with 2·0 % of Parke Davis, Witte and Morson's peptones were examined; referring the surface tension to dynes per cm. the following results were obtained:

Table I.

Beef	Peptone	Surface tension in dynes per cm.
Imported	Witte	53·4
,,	Parke Davis	58·2
,,	Morson	59·6
English	Witte	53·3
,,	Parke Davis	58·0
,,	Morson	59·6

These results agree fairly closely with those of Larson, Cantwell and Hartzell who found the surface tension of their media to be between 58 and 60 dynes per cm. The principal factor determining the surface tension of the medium appears to be the peptones, and of those used in the present experiments Witte's appears to have the greatest effect. In order to test this, 1·0 % solutions of the three peptones were made up, the specific gravity and surface tension were determined and the effect of equalising the hydrogen ion concentration ascertained. The results were as follows:

Peptone	S.G.	Surface tension dynes per cm.	p_H	Surface tension dynes per cm. at p_H 7·6
Witte	1·006	54·6	6·6	54·2
Parke Davis	1·005	61·5	6·3	62·1
Morson	1·006	60·5	5·6	60·6

It will be seen that the change produced by reducing the hydrogen ion concentration of the peptone solutions to a common number was slight.

In order to examine the effect of sodium ricinoleate on a nutrient broth, the 5·0 % solution of this salt was progressively diluted and the surface tension of the dilutions determined. They were as follows:

Table II.

Dilution	Sodium ricinoleate %	Surface tension dynes per cm.
0	5·0	32·8
4/10	2·0	33·6
1/10	0·5	39·7
1/25	0·2	47·0
1/50	0·1	52·0
1/100	0·05	57·0

As the surface tension of the peptone broth was already between 55 and 60 dynes per cm., it was decided to use much higher dilutions of sodium ricinoleate. The concentration of the soap in the medium is given in the following table and the surface tension before and after sterilisation:

Table III.

Na ricinoleate %	Surface tension dynes per cm.	
	Before sterilisation	After sterilisation
0·125	40·6	41·2
0·062	43·0	43·4
0·031	48·1	48·4
0·015	51·5	51·6
0·0	59·5	59·2

It will be observed that any hydrolysis of the soap which may have occurred as the result of heating to a high temperature did not measurably affect the surface tension of the media.

The media given in the above table were taken in lots of 10 cc., sterilised

and inoculated with the following rapidly growing organisms: *B. coli*, *C. diphtheriae*, *B. pyocyaneus*, *B. proteus*, *Vibrion septique*, *B. welchii* and *B. subtilis*, and examined after 48 hours' incubation at 37°.

Table IV.

Organism	Concentration of Na ricinoleate %				
	0	0·015	0·031	0·062	0·125
B. coli	+	+	+	+	+
C. diphtheriae	× × +	+ +	+	?	?
B. pyocyaneus	× × + +	× × + +	× × × +	× × +	× × +
B. proteus	× × + +	× + +	× + +	× + +	× + +
V. septique	× + +	+	?	?	?
B. welchii	+	+	?	?	?
B. subtilis	+	+	?	?	?

Note. × represents pellicle formation; + represents growth.

As will be seen, *B. coli* appears to be relatively indifferent to a concentration of soap which is definitely inhibitory to the anaerobes. Among the more affected is *C. diphtheriae*. This is in agreement with the statements arrived at empirically regarding the necessity of removing all fats from the medium if the growth of this organism is to be satisfactory.

By incubating the cultures for a further 24 hours the differences were accentuated.

Table V.

Organism	Concentration of Na ricinoleate %				
	0	0·015	0·031	0·062	0·125
B. coli	+ +	+ +	+ +	+ +	+ +
C. diphtheriae	× × + +	× × + + +	+ +	+	?
B. pyocyaneus	× × + + +	× + + +	× × + +	× × + +	× × + +
B. proteus	× + +	× + + +	× × + +	× + +	× + +
V. septique	× +	× × + +	+	+	× +
B. welchii	× +	× + +	+	?	?
B. subtilis	× × × +	× × × + +	+	+	?

The only additional point of interest in this table is the apparently slight stimulation to growth seen with the lowest concentration of soap used.

This experiment was repeated except that *B. typhosus* was added to the series. *B. sporogenes* showed itself extremely sensitive to the action of soap. *B. typhosus* grew, but with lessened vigour, even in the more concentrated tubes of soap broth.

The next set of experiments was made to test the effect of soaps on glucose fermentation.

For this purpose peptone broth containing varying quantities of sodium ricinoleate was tubed in portions of 10 cc. and sterilised. Each tube contained a Durham tube. To each tube after autoclaving was added 0·2 cc. of a sterile 10 % glucose solution with a sterile pipette. The tubes were placed in the incubator for 24 hours to control their sterility. The tubes were each inoculated with a loopful of actively growing culture and placed in the incubator at 37°.

The results at the end of 24 hours are given in the following tables:

Table VI.

	Concentration of sodium ricinoleate %				
	0	0·035	0·052	0·104	0·156
	Time—24 hours.				
B. paratyphosus B	+ +0ggg	+ +0g	+ +0gg	+ +0gg	+ +0ggg
B. subtilis	+ + +pp0	000	000	000	000
B. welchii	+ +0ggg	000	000	000	000
C. diphtheriae	000	000	000	000	000
B. muriseptícus	+ +pp0	000	000	000	000
B. paratyphosus A	+ +0ggg	+ +0gg	+ +0gg	+ +0g	+ +0gg
B. sporogenes	+ +0gg	000	000	000	000
B. coli	+ + +p(?)g	+ +p(?)g	+ +p(?)g	+ +p(?)g	+ +p(?)g
B. pyocyaneus	+ +pp0	+ +pp0	+ +pp0	+ +pp0	+ +pp0
B. typhosus	+ +00	+ +00	+ +00	+ +00	+ +00
B. proteus	+ +0gg	+ +0g	+ +0gg	+ +0g	+ +0g
	Time—48 hours.				
B. paratyphosus B	+ + +pggg	+ +0g	+ +0gg	+ +0gg	+ +0gg
B. subtilis	+ + +pp0	+p0	+00	000	000
B. welchii	+ + +0ggg	000	+0g	000	000
C. diphtheriae	+p0	000	000	000	000
B. muriseptícus	+ + +p0	+ +p0	000	000	000
B. paratyphosus A	+ +0gg	+ +0gg	+ +0gg	+ +0gg	+ +0gg
B. sporogenes	+ + +0g	+ +0g	+0g	+0g	000
B. coli	+ +0gg	+ +pgg	+ +0gg	+ +0gg	+ +0gg
B. pyocyaneus	+ +p0	+ +p0	+ +p0	+ +p0	+ +p0
B. typhosus	+ +00	+ +00	+ +00	+ +00	+ +00
B. proteus	+ +0gg	+ +0gg	+ +0gg	+ +0gg	+ +0gg
	Time—96 hours.				
B. paratyphosus B	+ +0gg	+ +0g	+ +0gg	+ +0gg	+ +0gg
B. subtilis	+ + +pp0	+ +pp0	+ +pp0	000	000
B. welchii	+ +0ggg	000	+0g	000	+?00
C. diphtheriae	+ +pp0	000	000	000	000
B. muriseptícus	+ + +pp0	+ +pp0	+00	000	000
B. paratyphosus A	+ +0gg	+ +0gg	+ +0gg	+ +0gg	+ +0gg
B. sporogenes	+ +0gg	+ +0gg	+ +0gg	+ +0gg	+00
B. coli	+ + +0gg	+ + +ppgg	+ + +ppgg	+ + +0gg	+ + +pgg
B. pyocyaneus	+ +pp0	+ +pp0	+ +pp0	+ +pp0	+ +pp0
B. typhosus	+ +00	+ +00	+ +00	+ +00	+ +00
B. proteus	+ +0gg	+ +0g	+ +0gg	+ +0gg	+ +0g

Note. The signs used are + =growth, *p* =pellicle, *g* =gas. Where 0 occurs the respective characteristic is absent.

It will be noted that among those bacteria which are most susceptible to the action of the ricinoleate are *B. welchii* and *B. subtilis*. Organisms flourishing in the intestine do not seem to be at all susceptible even to large quantities of soap, confirming similar observations of Larson [1922].

In view of the susceptibility of some of these organisms to quite small quantities of soap it was decided to repeat the above experiment, using smaller concentrations, the amount of glucose employed being the same. Only the 24 hours' and 96 hours' results are recorded here.

Table VII.

	Concentration of sodium ricinoleate %				
	0	0·012	0·025	0·050	0·075
	Time—24 hours.				
B. paratyphosus B	× ×0*gg*	× ×0*gg*	× ×0*gg*	× ×0*gg*	× ×0*gg*
B. subtilis	× ×*pp*0	× ×*pp*0	× ×*pp*0	× ×*pp*0	000
C. diphtheriae	× ×*pp*0	000	000	000	000
B. murisepticus	× ×*pp*0	× ×*pp*0	× ×*pp*0	× ×*pp*0	× ×*pp*0
B. paratyphosus A	× ×0*gg*	× ×0*gg*	× ×0*gg*	× ×0*gg*	× ×0*g*
B. coli	× ×*ppg*	× ×*ppg*	× ×*pg*	× ×*ppg*	× ×*ppg*
B. pyocyaneus	× ×*pp*0	× ×*pp*0	× ×*pp*0	× ×*pp*0	× ×*pp*0
B. typhosus	× ×00	× ×00	× ×00	× ×00	× ×00
B. proteus	× ×0*gg*	× ×0*g*	× ×0*g*	× ×0*gg*	× ×0*g*
B. enteritidis	× ×*ppgg*	× ×0*gg*	× ×0*g*	× ×0*g*	× ×0*gg*
	Time—96 hours.				
B. paratyphosus B	× ×0*gg*	× ×0*gg*	× ×0*gg*	× ×0*gg*	× ×0*gg*
B. subtilis	× ×*pp*0	× ×*pp*0	× ×*pp*0	× ×*pp*0	× ×*ppp*0
C. diphtheriae	× ×*pp*0	×*pp*0	000	000	000
B. murisepticus	× ×*pp*0	× × ×*pp*0	× × ×*pp*0	× ×*pp*0	× ×*pp*0
B. paratyphosus A	× ×*ogg*	× ×0*gg*	× ×0*gg*	× ×0*gg*	× ×0*gg*
B. coli	× ×0*gg*	× ×0*gg*	× ×*pgg*	× ×0*g*	× ×0*g*
B. pyocyaneus	× ×*pp*0	× ×*pp*0	× ×*pp*0	× ×*pp*0	× ×*pp*0
B. typhosus	× ×00	× ×00	× ×00	× ×00	× ×00
B. proteus	× ×0*gg*	× ×0*g*	× ×0*g*	× ×0*g*	× ×0*g*
B. enteritidis	× ×0*gg*	× ×0*gg*	× ×0*gg*	× ×0*g*	× ×0*g*

It will be noted that here again *C. diphtheriae* shows itself unusually susceptible to small concentrations of soaps.

The next series of experiments was directed towards ascertaining whether changes in the surface tension of the medium took place during growth of *C. diphtheriae*. The reason for this was partly the interesting statements of Warden and his co-workers [1921] on the constitution of toxins in general. If toxin production be contingent on the dispersion of fatty acids on certain colloid aggregates, then one would anticipate that changes would occur in the surface tension of the medium during the transfer. Furthermore, it might be expected that during the early growth of the bacteria and the consequent introduction of large colloidal particles, the surface tension-lowering substances already present in the medium would be adsorbed by the bacteria with a rise in their surface tension.

In order to get better results than had been obtained with the original form of Traube stalagmometer it was decided to use a drop weight method with a much slower rate of drop and to weigh the drops as formed. In the first set of experiments 30 drops were weighed and these were collected in a small weighing bottle which enclosed the tip of the stalagmometer. In this way disturbances from currents of air and from evaporation of the liquid were avoided. The total time for dropping was $3\frac{1}{2}$ minutes. The average weight of a drop of water from this tip was 0·105 g.

The results with this method were very satisfactory from a comparative point of view, quadruplicate weighings of 30 drops of water did not differ by more than 0·2 %. With peptone solutions the results were not so concordant, a difference of 1·0 % being obtained.

A series of very careful experiments was then made in which five drops of the medium were weighed, the time taken for a single drop of water to fall being about 45 seconds. Excellent results were given by the slow method of drop, and as was to be expected the values for surface tension approximated to those given by the static method when peptone solutions were tested. The differences obtained did not appear to justify the employment of the slower method of drop.

A series of tubes of bouillon peptone was set up and inoculated with the pellicle of a rapidly growing culture of *C. diphtheriae*. At stated times a tube of the culture was taken from the incubator, the culture centrifuged and the clear liquid examined. The results were as follows:

Hours	Surface tension dynes per cm.
control	57·8
24	58·2
72	58·9
120	55·8
168	58·2

It will thus be seen that during the early period of growth the surface tension rose owing to the adsorption by the bacteria of surface tension-lowering substances in the original medium. Following this there is a discharge of these materials into the medium, due, probably, to death and autolysis of bacteria, and the surface tension falls. The last determination gives a high value, but whether this is associated with other changes in the medium cannot be stated.

A second experiment was made in which not only the surface tension was determined, but at the same time the reaction and the production of carbonates were estimated. In this experiment quantities of bouillon of 100 cc. were inoculated. These were contained in flat medicine bottles which were incubated lying on their larger sides. The surface of medium exposed to the air was about 115 cm. The results were as follows:

Table VIII.

Hours	p_H	CO_2 from 1 cc. medium by acid	Surface tension dynes per cm.
0	8·0	0·09	60·06
72	8·4	0·27	62·20
120	8·4	0·29	62·00
168	8·5	0·30	61·37
240	8·5	0·34	62·70
336	8·6	0·34	62·99
432	8·5	0·32	62·41
768	8·5	0·30	62·90

The results of this experiment run quite differently from the preceding one. In the former it was noted that the surface tension of the medium rose slowly after inoculation to fall later. In the present series the rise occurs but the surface tension of the medium does not fall. The results in both series are the average of very closely agreeing duplicate determinations. In the second series, the surface exposed and the amount of pellicle formed is much greater

than in the test-tube series. This would tend to give a much larger amount of pellicle and leave a greater effective surface for the adsorption of surface tension-lowering substances.

In order to obviate some of the difficulties of the dynamic method it was decided to devise an apparatus which would give static readings. It will not be necessary to mention the various types which are already described in the literature some of which are known to give a high degree of accuracy when properly used.

It seemed that the pull given by a platinum ring would, on account of the ease of cleaning, be the most convenient for the purpose. This method has quite recently been used by du Nouy [1919] who obtained an increasing pull by utilising a torsion wire as had been done by Searle. The apparatus of du Nouy is a very expensive one and it seemed possible that by utilising the device of a dividing chain and ordinary hand scales one could produce the same effect with rather more gradual increase in weight than is afforded by the torsion wire. The essential features of the apparatus (Fig. 1)[1] are a small hand balance (*A*) with arms 8 cm. in length, turning with a load of 2 mg., and a platinum ring (*G*) 0·75 mm. thick, 3·1 cm. in outside diameter, supported from a small platinum ring by three very thin platinum wires. The ends of the rings were soldered together with silver. Care was taken to have the larger ring perfectly flat and in subsequent handling all tendency to deform it was avoided. This was done by always removing the ring with a stout hooked wire fastened to a handle. When not in use the ring was kept in a wide mouthed bottle containing strong sulphuric acid and potassium dichromate. The ring was washed in tap water before use and gently dried over a flame. The rest of the apparatus was made up of "Meccano" parts.

The arrangement of the apparatus was as follows:

The balance was suspended from a framework (*B*) 42 cm. high and 30 cm. wide at the base. The frame was slightly sprung inwards at the top and suitably braced to increase its rigidity. Holes were drilled in the horn pans (*C* and *D*) and from the right hand pan a small gold coated eyeglass chain (*F*) fell which ended in a hook supporting the platinum ring (*G*).

From the other fell a much longer (*E*) chain which was doubled upwards and was fastened to a hook which could be made to travel up and down by means of a rack and pinion (*H*). The position of the hook was determined by a fixed millimeter scale (*L*). By lowering the hook, an increase in weight, due to increase in the pan portion of the chain, was put upon the pan. By adding weights to the right hand pan (*C*) it was easy to construct a curve giving the increments of weight on the millimeter scale. In the present apparatus one millimeter roughly corresponded to 2 mg.

An improvement to the apparatus as drawn consisted in gearing down the rate of rotation of the wheel by means of a second smaller wheel fixed near

[1] I am indebted to Mr Colbrand of the Engineering School for the isometric scale drawing of the apparatus.

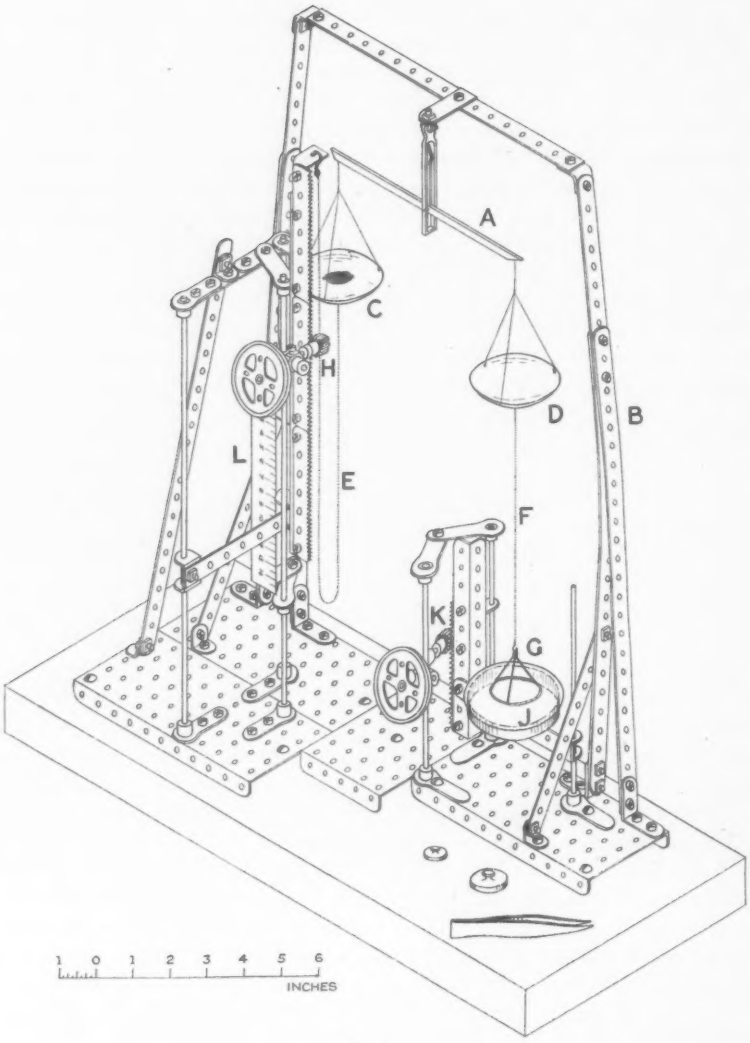

Fig. 1

the bottom of the pillar and connected with the former by a small catgut driving cord. The handle of this spindle was made from the largest sized meccano wheel. This slight alteration is a considerable improvement in the smoothness of movement of the ratchet.

The vessel used for holding the liquid to be examined was a large watch glass (*J*). This was supported on a platform which could also be raised and lowered by a rack and pinion (*K*) so that when the platinum ring was immersed in the fluid, after being balanced without contact, the balance could be returned to the zero point. The movable platform allowed the liquid to be raised gently until contact with the platinum ring was secured.

All determinations were made at room temperature which was 16–18°. For more precise work the container or the whole apparatus could be enclosed in a simple thermostat. Using fresh tap water to determine the water constant of the apparatus the weight required to tear the ring from the surface was 1·698 g.

The method of making a determination was as follows.

The scales were balanced by shot so that the pointer for the millimeter scale was at zero. The watch glass was provided with a few cc. of the solution to be examined, and was slowly moved up until the platinum ring was just in contact with the surface of the fluid. Weights were then added until the ring was being pulled away from the liquid without breaking contact. The weights were gently placed on the scale pan with as little disturbance as possible. With a little experience one easily recognises when it is time to have recourse to the chain for finally increasing the weight. At this point, by means of the rack and pinion on the left, the chain weight is *gradually* lowered. This should be done with as even and slow a motion as possible. At a certain point the ring is torn from the liquid and readings of the weights in the pan and on the millimeter scale are noted, and the scale reading converted into milligrams from the plotted curve. This is added to the pan weights and from the total weight the surface tension of the liquid is deduced. The experiment is then repeated to ensure concordance. With a total weight of over 1 g., individual experiments do not vary much more than 5–7 mg., so that the apparatus acts with an accuracy well under 1 %. As was to be expected the values obtained for the surface tension of solutions of peptones were considerably below those obtained by the ordinary drop methods. On an average the values were about 7–10 dynes per cm. lower. This difference was almost entirely due to a function of the dropping rate of the stalagmometer, for the experiments with the drop weight method in which the drops were produced at a very slow rate gave results very close to those obtained with the static method.

An experiment with *C. diphtheriae* will indicate to what extent growth of the organism in a peptone broth medium affects the surface tension. The strain used was P.W. 8 growing actively in the same medium. The culture used for inoculation was two days old. These experiments were performed in test-tubes.

The initial surface tension of the medium was 50·2 dynes per cm.; the p_H 7·8.

The following table gives the curve of the surface tension as measured by the static method:

Table IX.

Hours	p_H	Surface tension in dynes per cm.
0	7·8	50·2
24	—	52·2
48	6·8	47·1
72	7·5	45·7
120	8·2	47·6
144	8·5	46·9
192	8·5	45·6
240	8·5	45·2
288	8·5	45·6

This table also gives the interesting result that during the very early stage of growth some of the substances which produce a fall in surface tension are apparently adsorbed on the bodies of the young bacteria and therefore the surface tension rises. With death and autolysis, which occur later, fatty substances are liberated and with this the tension falls. It is noteworthy that this is not coincident with an alkaline reaction of the medium, for at the 48th hour, when the nutrient broth had a reaction of p_H 6·8, the tension dropped to 47·1 dynes per cm. It thus agrees with the first experiment in which the surface tension was determined by the drop weight method. This experiment was also performed with test-tube cultures.

The differences due to the media being contained in deep layers in test-tubes or in shallow layers with a larger surface exposed to air were so striking, that it was decided to repeat them in shallow layers on a larger scale and to use for comparison strains which were known to be toxigenic and those which were non-toxigenic. There were also drawn into the investigation three media which experience has shown to be the most suitable for the production of toxin.

Two strains of non-toxigenic *C. diphtheriae* were used, which were obtained from the National Type Collection at the Lister Institute. The toxigenic strain was P. W. No. 8. Two cultures of this strain were used, one of which had been subcultured in this laboratory for some years, the other being a fresh culture from the Lister Institute. No difference in behaviour between the two cultures was noticed. The three media employed were (1) the ordinary peptone broth, (2) Hartley's [1922] modification of Douglas's medium and (3) Martin's medium made up in accordance with the direction of Abt and Loiseau [1922].

The media were sterilised in 1300 cc. Fernbach flasks containing 500 cc. of medium. The necks of the flask were plugged with cotton wool. Through the neck passed a glass tube the upper part of which was bent at a right angle. This was provided with a rubber tube and screw clip. By attaching the rubber tube to a small wash bottle and gently sucking, a small amount of the

contents of the Fernbach flask could be removed without disturbing the flask in the incubator. The samples drawn off were usually so clear that it was not necessary to centrifuge them. The device is similar to that employed by Wolf and Harris [1917] in their study of anaerobic fermentation except that the cultures are maintained aerobically.

Only sufficient of the medium was withdrawn to determine the hydrogen ion concentration and the surface tension. At the end of the experiment the culture was examined for contamination. None such was ever found. Only one of the three experiments will be given although the qualitative results were very similar.

Table X.

	Medium											
	Martin				Douglas				Peptone broth			
	P.W. No. 8		Gentry		P.W. No. 8		Gentry		P.W. No. 8		Gentry	
Hours	p_H	S.T.	p_H	S.T.	p_H	S.T.	p_H	S.T.	p_H	S.T.	p_H	S.T.
0	7·5	44·8	7·5	44·9	7·5	46·6	7·5	45·7	7·6	44·9	7·6	45·7
24	7·2	45·6	7·1	48·6	7·0	48·0	7·0	48·1	6·8	47·8	6·6	48·3
48	7·4	47·0	7·2	49·1	7·2	48·0	7·3	49·6	6·6	48·4	6·8	50·3
72	8·0	48·3	7·6	52·8	7·7	49·0	7·8	53·0	7·2	48·6	7·5	54·1
96	8·3	49·2	8·2	54·0	8·0	50·0	8·0	53·1	7·7	49·0	8·0	54·7
120	8·4	50·2	8·4	55·1	8·2	51·6	8·2	52·6	8·0	51·3	8·2	55·4
144	8·4	51·1	8·5	55·4	8·2	52·0	8·4	54·6	8·2	51·6	8·4	57·8
168	8·4	51·6	8·5	54·9	8·2	51·9	8·4	54·1	8·1	51·2	8·5	57·4
264	8·5	52·6	8·6	57·8	8·4	54·0	8·6	55·7	8·4	53·7	8·7	58·6
384	8·5	52·8	8·7	57·5	8·6	52·8	8·6	53·8	8·6	51·6	8·7	58·2
576	8·8	54·8	8·8	55·3	8·7	53·3	8·8	54·3	8·8	51·6	8·8	57·9

The main point to be observed is the continuous rise in the surface tension of the medium during the growth of the culture and what is more important during a time when it may be assumed that growth has practically ceased.

Speaking generally it may be assumed that the toxin production under the conditions of the experiment had reached its maximum at the fifth day. Curves were drawn of the rise in surface tension and it was found that this rise was, on the whole, steepest during the first 120 hours of the experiment. What is more striking is the divergence of the curves for a given medium produced by a toxigenic and a non-toxigenic strain. In each pair of experiments the curve of rise in surface tension given by a non-toxigenic strain is much steeper, and the ultimate rise in surface tension much higher than with a toxigenic bacillus. This would lead one to believe that the adsorption of surface tension-lowering substances by the bodies of the bacteria is more complete when little toxin is formed.

It may be that the envelope of the bacteria, by reason of its content in fat plays an important part in determining toxin production. Those strains of bacteria whose outermost coating has the greater amount of fat will not adsorb the metabolic products of surface tension-lowering capacity on which toxin production depends. That such substances are elaborated is indicated, not only by the experiments of Warden to which allusion has already been made, but also by the more recent investigations of Walbum [1923] and Doyon

[1922]. Walbum, in particular, believes that a non-toxic "protoxin" is elaborated by the bacillus, and this in contact with peptone or peptone broth produces the active toxin. One may take it that the "protoxin" is of a surface active character, and if adsorbed on the bodies of the bacteria would be removed from a sphere where toxin can be formed. This would give a partial explanation for the fact that non-toxic strains of bacteria remove the surface tension-lowering substances from the medium.

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C. THE CONSTITUTION OF GALEGINE.

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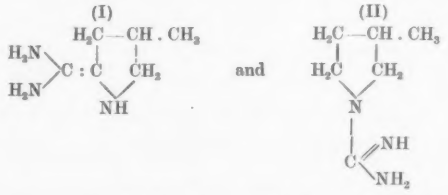
Tanret [1914] isolated from the seeds of *Galega officinalis* a base of the formula $C_6H_{13}N_3$, which is hydrolysed by baryta according to the equation:

$$C_6H_{13}N_3 + H_2O = CON_2H_4 + C_5H_{11}N$$

into urea and a volatile base; the latter Tanret (erroneously) regarded as 3-methylpyrrolidine, which led him to suggest two possible formulae for galegine:



Neither formula accounts for the properties of galegine, as given by Tanret. According to both, galegine would possess an asymmetric carbon atom; yet this base has no optical activity, nor could Tanret resolve it. Moreover, substances of either constitution would give monobenzoyl derivatives (or in the case of formula (I) possibly a tribenzoyl compound); yet Tanret obtained a *di*benzoyl derivative of galegine. These discrepancies induced us to re-investigate this question, particularly since the formation of urea by hydrolysis suggested that galegine is a guanidine derivative, and naturally occurring guanidine derivatives are rare. Tanret had indeed already considered its relationship to arginine (see note at the end of this paper).

We soon established the presence of a guanidine nucleus, because galegine gives Weyl's test and the diacetyl reaction [Harden and D. Norris, 1911] and is precipitated by silver nitrate in ammoniacal solution. The correct identification of the other fission product $C_5H_{11}N$ was a more lengthy process. Tanret had found that the boiling point of this base (105–108°) and the melting point of its platinichloride (194–196°) correspond closely to those recorded for 3-methylpyrrolidine (103–105° and 194–196° respectively); the agreement with the corresponding data for piperidine (B.P. 106°, M.P. of platinichloride 198–200°) is, however, almost as good. But when we had prepared the corresponding aurichloride and picrate, it was evident that the base was not identical with either 3-methylpyrrolidine or with piperidine. In order to choose between

the several other possible constitutions, a closer examination was necessary, which yielded the following results:

1. The volatile base gives Hofmann's carbylamine reaction.

2. Treated according to Hinsberg, with toluenesulphonyl chloride it gives a toluenesulphonamide soluble in sodium hydroxide.

3. It decolorises potassium permanganate in dilute sulphuric acid solution [Willstätter, 1900].

Observations 1 and 2 prove the base to be a primary one, observation 3 proves it to be unsaturated: it is therefore an amino-amylene.

In conformity with this view, galegine was found to take up exactly one molecule of hydrogen when reduced with palladium as catalyst, and the resulting dihydrogalegine yielded on distillation with lime *iso*-amylamine.

Now only the position of the double bond in galegine remained to be determined. This was done by oxidising galegine sulphate with barium permanganate, which yielded both acetone and glycocyamine (guanidino-acetic acid). The constitution was now almost established, for the oxidation may be expressed thus:

$$\begin{matrix}CH_3\\CH_2\end{matrix}\!\!>\!C:CH\,.\,CH_2\,.\,NH\,.\,C(:NH)NH_2 + 3O = \begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CO + HOOC\,.\,CH_2\,.\,NH\,.\,C(:NH)\,.\,NH_2.$$

We say "almost established" for in the analogous case of citronellal, it was shown by Harries and Schauwecker [1901], after much controversy, that the constitution is

$$\begin{matrix}CH_3\\CH_2\end{matrix}\!\!>\!C\,.\,CH_2\,.\,CH_2\,.\,CH_2\,.\,CH(CH_3)\,.\,CH_2\,.\,CHO$$

which does not prevent the formation of acetone [Tiemann and Schmidt, 1897]. It is a fine point, therefore, whether galegine has not the constitution

$$\begin{matrix}CH_3\\CH_2\end{matrix}\!\!>\!C\,.\,CH_2\,.\,CH_2\,.\,NH\,.\,C(:NH)\,.\,NH_2$$

instead of that given in the above equation. It may perhaps be possible to settle this by a similar method to that employed by Harries and Schauwecker [1901] for citronellal.

Boiled with dilute sulphuric acid, galegine becomes stable to permanganate and takes up a molecule of water, forming hydroxydihydrogalegine; probably the hydroxy group is attached to the tertiary carbon atom.

Although the constitutions of galegine and its hydration product have still slight elements of uncertainty, such uncertainty does not arise in the case of dihydrogalegine; its constitution was, moreover, placed beyond doubt by its synthesis from *iso*-amylamine and cyanamide. The analogous synthesis of galegine itself would require the unsaturated amine $C_5H_{11}N$; very few bases of this type are known, for their synthesis presents considerable difficulty. It is indeed remarkable how few unsaturated simple bases occur in nature. Galegine is even more interesting in this respect than in the possession of a guanidine nucleus.

Methods of isolating galegine from the seeds.

The seeds were obtained and extracted for us by the British Drug Houses, Ltd. for whose help we are much indebted. From a portion of the extract galegine sulphate was first isolated by following Tanret's directions exactly, but we found his elimination of acetic acid by ether and removal of the sugar very tedious and afterwards greatly simplified the process. The extract (obtained after evaporating the 60 % alcohol used in extraction) contained much fat and was ground up with sand to a stiff paste and extracted several times with cold water. The combined decantations and final filtrate were treated successively with normal and basic lead acetate, the lead was removed as sulphate, the excess of sulphate ions quantitatively by baryta, and the filtrate was evaporated to a thin syrup. On adding 50 % sulphuric acid until acid to Congo the galegine sulphate ultimately crystallised in a yield of 0·5 % of the seeds, the same as that obtained by Tanret. After we had found that galegine is precipitated by silver nitrate and ammonia, we worked up the mother liquors by Kossel and Kutscher's method for the isolation of arginine, but only obtained a very small additional amount. Galegine, being unsaturated, is even more readily oxidised by silver in alkaline solution than creatinine [cf. Ewins, 1916]. In any case the solubility of galegine sulphate in cold water is so small, that but little is lost in the mother liquors. We also used an alternative method of isolation by precipitation with potassium bismuth iodide, regenerating with lead hydroxide. The resulting syrup would not at once yield the sulphate; it indeed readily gave the picrate, but as the regeneration of the latter is troublesome (see below) this alternative method is not recommended.

Colour reactions of galegine.

(*a*) Weyl's reaction. 2 cc. of a very dilute solution of sodium nitroprusside is made alkaline with 2 drops of 10 % sodium hydroxide and one drop of a 1 % solution of galegine sulphate is added; after standing for 15 minutes a red coloration had developed; limit 1 : 6000.

(*b*) Diacetyl reaction. One drop of diacetyl (prepared according to Diels and Stephan [1907]) is dissolved in 5 cc. of water and 2 drops of 10 % sodium hydroxide are added. On further adding one drop of a 0·5 % solution of galegine sulphate and warming slightly, the characteristic pink coloration of guanidine derivatives is produced. Limit 1 : 10,000. Under similar conditions we found the limit for arginine nitrate 1 : 5000.

Reduction of galegine; dihydrogalegine and its salts.

0·88 g. galegine sulphate (5 millimols of base) was dissolved in 25 cc. of water, 0·02 g. palladium chloride in a little very dilute hydrochloric acid was added, and the solution was shaken in a hydrogen atmosphere under a pressure of 2 atmospheres. The absorption was complete in three hours; no gum arabic was necessary.

Hydrogen absorbed: 115 cc. at 11° and 752 mm. = 109 cc. at N.T.P.
Hydrogen calculated for 5 millimols 112 cc. ,,

The catalyst, which had coagulated, was filtered off, and the solution was evaporated to a syrup. On dissolving this in boiling alcohol and cooling, dihydrogalegine sulphate crystallised in colourless prisms, M.P. 270° (galegine sulphate melts at 227°).

0·2033 gave 0·1336 $BaSO_4$ = 27·6 % H_2SO_4; $(C_6H_{15}N_3)_2H_2SO_4$ = 27·2 % H_2SO_4.

The sulphate is only slightly soluble in alcohol and water, yet more so than galegine sulphate. Weyl's and the diacetyl reaction are similar to those with galegine, but less sensitive. Permanganate and bromine water are not decolorised.

Dihydrogalegine nitrate is little soluble in dilute nitric acid but fairly readily in water and alcohol, and forms long needles from alcohol and ether, M.P. 75–76°.

Dihydrogalegine picrate is almost insoluble in cold water and crystallises from hot water in long narrow plates, M.P. 172°.

1 g. of the dry sulphate was mixed with 4 g. of quick lime and distilled. Besides ammoniacal vapours, it yielded (1) a colourless liquid, B.P. 89–95°; (2) a small fraction, B.P. 95–105° from which minute crystals separated, resembling cholesterol; (3) a yellow viscous mass, remaining in the flask, partly sublimed and partly distilled at 175°/0 mm., forming an oily liquid which solidified to a partially crystalline wax. This was insoluble in dilute acids, but soluble in concentrated hydrochloric acid, from which it was precipitated by ammonia. It is also soluble in glacial acetic acid and yields a picrate, after the solution has been diluted by water. A similar substance has been observed by Tanret on distilling galegine. We mention its production in the present case as evidence that its formation is independent of the unsaturated linking. The feeble base, which is formed perhaps from any alkylguanidine, requires further investigation.

The first fraction of the distillate was redistilled twice over solid potassium hydroxide and then over a mixture of sodium and potassium when it boiled pretty constantly at 95–96°. It had a strong ammoniacal odour, fumed in air and readily formed a carbonate, gave a white precipitate with Nessler's reagent, was stable to dilute acid permanganate and gave the carbylamine reaction.

The hydrochloride (long thin plates from acetone) M.P. 215° and the picrate M.P. 130–134° were found to be identical with the corresponding salts prepared from commercial *iso*-amylamine. A mixture of the hydrochlorides melted at 214°, of the picrates at 130–133°. *Iso*-amylamine picrate was prepared by adding an ethereal solution of picric acid to the amine, and recrystallised, on standing, from an ethereal solution containing a little acetone. On rapidly evaporating the ethereal solution, this picrate was obtained in fine needles. The amine from dihydrogalegine is therefore *iso*-amylamine.

Synthesis of dihydrogalegine (iso-*amylguanidine*).

Following the method used by Kossel [1910] for the synthesis of agmatine, we kept a concentrated aqueous solution of 1 g. cyanamide and 1·9 g. *iso*-amylamine for ten days at room temperature. Unchanged *iso*-amylamine was then boiled off and a cold saturated solution of picric acid added. A voluminous precipitate was immediately formed; recrystallised from boiling water, it formed long narrow plates, similar to those of dihydrogalegine picrate; yield 2·1 g. = 27% of the theory. On further recrystallisation the picrate melted at 171° (dihydrogalegine picrate from galegine 172°). A mixture of the two specimens melted at 171–173°. The regeneration of this picrate, like that of galegine, was difficult on account of its very slight solubility and the fact that guanidine derivatives are powerful bases. Boiling with 10 % sulphuric acid only decomposed an insignificant proportion. The method finally adopted was to dissolve in warm glacial acetic acid, dilute with ether, and extract many times with dilute sulphuric acid. After washing the aqueous solution with ether and removing the sulphate ions quantitatively with baryta, a syrup resulted on evaporation, which on addition of nitric acid yielded the nitrate. This after recrystallisation melted at 75–76°, and, mixed with the nitrate of the reduction product of galegine, at 74–76°.

This further proves the identity of dihydrogalegine with *iso*-amylguanidine.

Oxidation of galegine.

Kutscher [1901] obtained guanidinobutyric acid by oxidising arginine with barium permanganate in alkaline solution. His yield was poor for most of the arginine was oxidised to guanidine. Since we wished to oxidise galegine at the double bond, we considered it better to use only a small quantity of permanganate and to work in acid solution.

0·88 g. galegine sulphate (= 5 millimols of base) was dissolved in 60 cc. of 5 % sulphuric acid and 1·88 g. barium permanganate (= 5 millimols) was added. This could at most yield 5 atoms of oxygen. The purple colour at once disappeared but, although the mixture was boiled, manganese dioxide remained undissolved, and only 3 atoms of oxygen were used up. The mixture of barium sulphate and manganese dioxide was filtered whilst hot, and the excess of sulphuric acid removed from the filtrate with barium carbonate. The solution was then concentrated to half its bulk by distillation, and the distillate kept to demonstrate the formation of acetone (see below). The solution was then evaporated to dryness on the water-bath, when a considerable amount of a solid remained which recrystallised from boiling water in acicular plates. On slow cooling these formed characteristic sheaves; they melted at 270–280°. The substance was little soluble in cold water but readily in both acids and alkalis. It gave the diacetyl reaction, but Weyl's reaction only very faintly and Jaffé's reaction not at all.

Micro-Kjeldahl: 6·21 mg. gave 2·25 mg. N = 36·2 %
4·50 mg. „ 1·64 mg. N = 36·4
Calculated for glycocyamine, $C_3H_7O_2N_3$ N = 35·9

For purposes of direct comparison we prepared some *glycocyamine* by heating chloroacetic acid with 5 molecular proportions of free guanidine in concentrated aqueous solution to 60° for two hours [Ramsay, 1908]. It was found to be identical with the oxidation product of galegine.

The hydrochlorides were prepared by evaporating a solution of the substance in hydrochloric acid, and formed colourless plates. M.P. (from galegine) 190°; synthetic 191°; mixture 189°.

The picrates formed long needles from hot water. M.P. (from galegine) 201–203°; synthetic 202°; mixture 201°.

As a further confirmation, a little of the oxidation product was heated with concentrated hydrochloric acid to 140° for two hours, so as to convert it into the anhydride, glycoamidine; the residue remaining on evaporation gave a deep blood-red colour with picric acid and sodium hydroxide (Jaffé's reaction).

Acetone was identified as the other oxidation product in the distillate obtained after removal of the sulphuric acid (above). It gave Legal's test with sodium nitroprusside and at once yielded a precipitate with *p*-nitrophenylhydrazine in 50 % acetic acid. The hydrazone crystallised from dilute alcohol in fine yellow needles, M.P. 148–149°. The melting point of the *p*-nitrophenylhydrazone of acetone is given as 148–148·5°. The yield was 0·49 g. or 50% of the theory.

The unsaturated amine $C_5H_{11}N$ *from galegine.*

As this amine was unknown, we had no material for direct comparison, and on account of the small amount available, we have not yet examined it very closely. We found it most convenient to prepare it directly from galegine sulphate by distillation with lime, as described above for dihydrogalegine; distilled over potassium hydroxide the amine boiled at 105–108°, as in Tanret's experiments; distillation over potassium did not, however, give a sharper boiling point, as it did in the case of *iso*-amylamine. This is evidently connected with unsaturation: potassium partially polymerised the product.

The amine is a primary base (carbylamine reaction; toluenesulphonamide soluble in alkali). It is unsaturated, at once decolorising permanganate and bromine water. It has a pungent, piperidine-like odour.

A rough determination of the density on the small amount of approximately pure distillate gave

$$D\,\frac{18°}{18°} = 0{\cdot}779.$$

Although this determination is not accurate, it suffices to establish the fact that the amine is unsaturated and not cyclic. The value $D^{18°}_{18°} = 0{\cdot}779$ is much (about 0·06) lower than that of cyclic amines of the formula $C_5H_{11}N$. Thus piperidine has $D^{18·6°} = 0{\cdot}8619$ and 3-methylpyrrolidine has $D^{0°}_{4°} = 0{\cdot}8654$. On the other hand the density found is slightly higher than that of the corresponding saturated *iso*-amylamine, from which it differs by the same amount as the densities of an amino-hexylene and the corresponding saturated hexy-

lamine. From the densities given in Beilstein (4th ed.) we calculate for *iso*-amylamine $D^{18°}_{18°} = 0{\cdot}753$, whence increment for a double bond would be 0·026. The density of $CH_2 : CH . CH_2 . CH_2 . CH(CH_3) . NH_2$ is given as $D^{15°}$ 0·779, of $CH_3 . CH_2 . CH_2 . CH_2 . CH(CH_3) . NH_2$ as $D^{20°}_{0°} = 0{\cdot}753$, whence a slightly smaller increment for the double bond may be deduced, corresponding to the higher position in the homologous series. For the much lower members of the series, allylamine ($D^{18°} = 0{\cdot}769$ approx.) and propylamine ($D^{18°} = 0{\cdot}720$ approx.) the increment is about 0·049.

In order to obtain the hydrochloride, a solution free from excess acid must be evaporated (alkaline to methyl red, but not to phenolphthalein). Excess of hydrochloric acid fixes itself on the double bond. The hydrochloride is somewhat hygroscopic, and very soluble. It was not analysed, but the following salts were prepared:

Platinichloride, from the solution of the amine in excess of hydrochloric acid, by adding aqueous platinic chloride solution. The yellow precipitate which separates at once, is crystallised from hot water; M.P. 194–197°.

41·9 mg. gave 14·13 mg. Pt	Pt = 33·7 %
Calculated for $(C_5H_{11}N)_2 . H_2PtCl_6$	Pt = 33·6 %

The same salt was obtained from the amine prepared according to Tanret, by hydrolysis of galegine by baryta at 100° and extraction with ether.

Aurichloride, from concentrated aqueous solutions of the hydrochloride and auric chloride. Rhomb-shaped plates from warm water, M.P. 57°. After drying *in vacuo* over sulphuric acid it sintered at 75° and melted at 81°. It was then analysed.

34·28 mg. gave 16·05 mg. Au	Au = 46·8 %
Calculated for $C_5H_{11}N . HAuCl_4$	Au = 46·35 %

Picrate. Ethereal solutions of the amine and of picric acid are mixed. It is moderately soluble in hot water, from which it crystallises in long narrow plates, M.P. 138·5–139·5°.

Action of hydrobromic and hydrochloric acids on the amine.

In an attempt to prepare the hydrobromide, the amine was evaporated to dryness with excess of hydrobromic acid. The residue, after drying over potassium hydroxide, crystallised from acetone, after adding ether, in fine colourless needles. The salt was not hygroscopic, but unstable to air and light and decomposed on rapid heating to 110°. It was therefore dried *in vacuo* at 78° over phosphorus pentoxide, when it melted at 206°.

Micro-Kjeldahl: 27·15 mg. gave 1·59 mg. N	N = 5·86 %
Calculated for $C_5H_{12}N . Br . HBr$	N = 5·67 %

Bromine estimation by direct precipitation with $AgNO_3$

0·1522 gave 0·2287 AgBr	Br = 63·9 %
Calculated for $C_5H_{12}N . Br . HBr$ for both bromine atoms	Br = 64·8 %

The salt is evidently the hydrobromide of a bromo-*iso*-amylamine which readily loses two molecules of hydrobromic acid. Whether the original un-

saturated amino-amylene is thereby reformed, or whether a new cyclic base results, has not yet been determined. In any case this does not appear to be methylpyrrolidine, since the bromo-*iso*-amylamine is not stable to permanganate in sulphuric acid solution.

Similarly chloro-*iso*-amylamine is obtainable by evaporating amino-amylene with hydrochloric acid. The hydrochloride of this chloro-amine is hygroscopic. It was converted into *the platinichloride* $(C_5H_{12}NCl)_2 . H_2PtCl_6$. This is much more soluble than the platinichloride of the unsaturated base, and is decomposed on boiling its aqueous solution. From dilute alcohol it formed iridescent six-sided plates, quite different in shape from the corresponding unsaturated salt, but having the same melting point (193–195°).

This salt decomposes at 110° and was therefore dried *in vacuo*.

35·13 mg. gave 10·88 mg. Pt	Pt = 31·0 %
23·93 mg. ,, 7·5 mg. Pt	Pt = 30·9 %
Calculated for $(C_5H_{12}NCl)_2 . H_2PtCl_6$	Pt = 29·9 %

Evidently this salt had already lost some hydrochloric acid, although the platinum content was still much below that of the unsaturated salt (33·6 %).

The aurichloride crystallises from warm water in plates, sintering at 90° and decomposing at 99–101°. It is also unstable, but was obtained practically pure by drying over sulphuric acid.

20·7 mg. gave 8·76 mg. Au	Au = 42·3 %
Calculated for $C_5H_{12}NCl . HAuCl_4$	Au = 42·7 %

The picrate formed by adding picric acid to the hydrochloride is very much more soluble than the unsaturated salt in water, alcohol or acetone. Washed free from picric acid with ether, it melted at the same temperature as the unsaturated picrate (137–138°) but contained chlorine.

Hydroxydihydrogalegine.

When converting a quantity of (the very slightly soluble) galegine picrate into the sulphate, the former was boiled for some time with dilute sulphuric acid, in order to dissolve it. The resulting sulphate was, however, very soluble in water, in contradistinction to galegine sulphate. It crystallised from methyl alcohol in fern-like crystals melting at 205–206°. (Galegine sulphate M.P. 227°; the mixture melted at 200°.)

The new sulphate was quite stable to potassium permanganate and to bromine water; it did not take up hydrogen in the presence of palladium, but it still gave Weyl's and the diacetyl reaction. The air-dry substance contains one molecule of water of crystallisation.

Dried at 120–130° it lost 4·1 %.

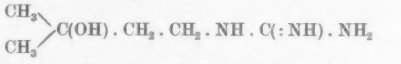
$(C_6H_{15}ON_3)_2 . H_2SO_4 . H_2O$ requires H_2O = 4·4 %.

The anhydrous substance was analysed.

0·2300 gave 0·3091 CO_2 and 0·1696 H_2O	C = 36·65; H = 8·2.
8·69 mg. (micro-Kjeldahl) gave 1·89 mg. N	N = 21·7.
0·2147 gave 0·1300 $BaSO_4$	H_2SO_4 = 25·4.

Calculated for $(C_6H_{15}ON_3)_2 . H_2SO_4$; C = 37·1, H = 8·0, N = 21·6, H_2SO_4 = 25·3.

On boiling with dilute sulphuric acid galegine therefore takes up a molecule of water, to form hydroxydihydrogalegine of the probable constitution:



The transformation can be conveniently followed by potassium permanganate. Boiling with 25 % sulphuric acid renders it complete in five minutes; with 5 % sulphuric acid about 45 minutes are required.

Hydroxydihydrogalegine picrate separates from a concentrated hot aqueous solution in rhomb-shaped crystals, M.P. 153–154°, much more soluble than galegine picrate.

In order to regenerate galegine from the picrate it is therefore necessary to avoid boiling with mineral acids. It can be done by dissolving in alcohol, diluting with much ether and extracting with successive small quantities of 50 % sulphuric acid. The aqueous solution is washed with ether and the sulphuric acid is removed quantitatively by baryta; the yield is unsatisfactory.

Hydroxydihydrogalegine yields on hydrolysis a hydroxy-*iso*-amylamine, which can also be obtained by boiling the unsaturated amine with sulphuric acid, but has not been examined further as yet. Its synthesis is being attempted, with a view to that of galegine.

This investigation was rendered possible by a grant to one of us (F.D.W.) from the Department of Scientific and Industrial Research. The cost of material and its extraction was met by a grant of the Moray Research Fund of this University. For both grants we wish to express our gratitude.

[*Note added November 22nd.*] This paper was submitted in MS. to M. Georges Tanret, who declared himself in agreement with our conclusions and sent us a copy of a thesis of the University of Paris, 1917, entitled *Recherches chimiques et physiologiques sur la graine de Galega*, in which (p. 34) he had considered in a footnote the possibility that galegine is a guanidyl derivative of an unsaturated *iso*-amylamine; he considered that on hydrolysis with baryta the amine is isomerised to 3-methylpyrrolidine. As we have shown, this latter supposition is incorrect.

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CI. GALUTEOLIN, A NEW GLUCOSIDE FROM *GALEGA OFFICINALIS.*

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During the preparation of galegine from the seeds of *Galega officinalis* (see the preceding paper) a yellow colouring matter was detected and identified as a new glucoside of luteolin, for which the name *galuteolin* is suggested. It gave a green coloration with ferric chloride, characteristic of catechol derivatives, and was accordingly precipitated by normal lead acetate in the preliminary purification for the isolation of galegine.

Isolation. The precipitate with normal lead acetate, obtained from the extract of six kilos. of seeds, was air-dried, finely ground and extracted several times with boiling dilute acetic acid, in which it was almost wholly soluble. After removal of the lead by hydrogen sulphide and concentration of the filtrate to a small bulk, a yellow solid separated, in a yield of 2 g. This was insoluble in water, and very slightly soluble in absolute alcohol, but more so in hot dilute alcohol from which it crystallised in aggregates of yellow needles melting with decomposition at 280°. These crystals gave the original olive green coloration with ferric chloride, and a yellow precipitate with normal lead acetate, soluble in hot dilute acetic acid. They reduced Fehling's solution and ammoniacal silver nitrate, and dissolved in alkalis with a deep yellow colour. On boiling a small portion with dilute sulphuric acid, cooling and filtering, the filtrate gave Molisch's reaction, suggesting that the colouring matter was a glucoside.

Galuteolin crystallises with 3 molecules of water, of which $2\frac{1}{2}$ are lost at 120–130°, but the remaining $\frac{1}{2}H_2O$ is only removed with great difficulty. This behaviour is similar to that of the glucosides osyretin and violaquercitrin recorded by A. G. Perkin [1902] which both lose $2\frac{1}{2}H_2O$ at 130° but the remaining $\frac{1}{2}H_2O$ only at 160°. As our glucoside was not stable at the latter temperature, we preferred to dry it at the temperature of boiling tetrachloroethane *in vacuo* over phosphorus pentoxide.

0·1254 air-dry lost 0·0111 at 120–130°;	$H_2O = 8{\cdot}85$ %.
Calculated for $C_{21}H_{20}O_{11} . 3H_2O$	$2\frac{1}{2}H_2O = 8{\cdot}96$.
0·1137, dried as above, lost 0·0022 *in vacuo* over P_2O_5;	$H_2O = 1{\cdot}93$.
Calculated for $C_{21}H_{20}O_{11} . \frac{1}{2}H_2O$	$\frac{1}{2}H_2O = 1{\cdot}97$.

For analysis the glucoside dried at 120–130° was employed.

0·1480 gave 0·2978 CO_2 and 0·0594 H_2O; C = 54·9; H = 4·5%.
0·1538 „ 0·3104 CO_2 „ 0·0635 H_2O; C = 55·0; H = 4·6.
Calculated for $C_{21}H_{20}O_{11} \cdot \frac{1}{2}H_2O$ C = 55·1; H = 4·6.

A methoxy determination yielded a negative result.

Hydrolysis of galuteolin. This proceeds readily with quite dilute sulphuric acid [compare A. G. Perkin, 1909].

0·2904 (dried at 120°) was boiled with 20 cc. of 3 % sulphuric acid for 3 hours. The solution was cooled and the precipitate collected and dried; it weighed 0·1808 = 62·3 %.

Calculated for $C_{21}H_{20}O_{11} \cdot \frac{1}{2}H_2O$ $C_{15}H_{10}O_6 = 62·6$ %.

The hydrolysis is therefore represented by the equation:

$$C_{21}H_{20}O_{11} + H_2O = C_{15}H_{10}O_6 + C_6H_{12}O_6.$$

After removal of the sulphuric acid, the filtrate yielded a phenylosazone, which on recrystallisation from alcoholic pyridine melted at 203°. Mixed with a sample of phenylglucosazone it melted at 204°. The sugar is therefore glucose.

The greater part of the available glucoside was now hydrolysed, the resulting insoluble colouring matter crystallising from dilute alcohol in small needles of indefinite melting point, blackening at 290–300°.

After several crystallisations it was dried at 120° and analysed:

0·1305 gave 0·3002 CO_2 and 0·0451 H_2O; C = 62·7; H = 3·8 %.
0·1306 „ 0·3008 CO_2 „ 0·0463 H_2O; C = 62·8; H = 3·9.
Calculated for $C_{15}H_{10}O_6$ C = 62·9; H = 3·5.

The fission product is readily and completely acetylated by boiling for a minute with acetic anhydride and a drop of pyridine or trace of concentrated sulphuric acid. The acetyl compound, crystallised from absolute alcohol, forms long colourless needles, M.P. 221–223°.

0·1400 gave 0·3104 CO_2 and 0·0496 H_2O; C = 60·4; H = 3·9 %.
Calculated for $C_{15}H_6O_6(C_2H_3O)_4$ C = 60·8; H = 4·0.

The composition and melting point of the acetyl derivative suggested that the fission product was *luteolin*, and this identity was confirmed by comparison with a specimen of luteolin, for which we are indebted to the kindness of Prof. A. G. Perkin, F.R.S. A portion of the latter specimen was acetylated, and the acetyl derivative, from both sources as well as the mixture, melted at 221–223°.

Sublimation of hydroxyflavones. Before the definite identification of the fission product of our glucoside with luteolin we had already compared it with other hydroxyflavones by a method which does not seem to have been previously employed in this group. We found that in a high vacuum most hydroxyflavones may be sublimed without decomposition. We simply heated minute quantities in a test tube in a metal bath, evacuating with a rotary oil pump (pressure < 1 mm.). The temperature (of the bath) at which sub-

limation takes place, as well as the colour shade of the sublimate, is characteristic, for instance:

	Chrysin	Luteolin	Apigenin	Fisetin
Sublimes at	220°	250–270°	230–290°	230–330°
Resublimes at	220	230–235	260–270	280–290
Colour of sublimate	Almost white	Faint yellow white	Yellowish white	Brownish yellow

Since the melting points of hydroxyflavones are often very high their "mixed" melting points are unsatisfactory. Comparative sublimation (two tubes in the same bath) can be readily carried out with minute quantities.

We found that when galuteolin was similarly heated *in vacuo* a sublimate of luteolin appeared on the sides of the tube at the same temperature as before, and a tarry residue of decomposed sugar remained on the bottom.

In addition to galuteolin, luteolin itself was present in the extract of the seeds. Luteolin has been found previously in a number of plants (*Reseda luteola*, *Genista tinctoria*, *Digitalis purpurea*) but no glucoside of luteolin seems to have been isolated hitherto.

This research was made possible by a grant to one of us (F.D.W.) from the Department of Scientific and Industrial Research, for which grateful acknowledgment is made.

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CII. IMPROVEMENTS IN COLORIMETRY.

By ROBERT VINER STANFORD.

From the Chemical Laboratory, Cardiff City Mental Hospital.

(*Received September 20th, 1923.*)

Some years ago I pointed out [Stanford, 1913] that it is not possible to compare the depth of colour of two solutions unless they are of the same shade of colour, and that therefore the colorimetric comparison must be made when the liquids are of the same concentration, and this condition necessarily involves their being of the same thickness of layer. Following this principle, I described in that paper a "dilution" colorimeter, in which the two liquids are contained in similar glass cells, the stronger being then diluted until equality is reached. Whilst that instrument cannot well be criticised from a theoretical point of view, in actual use it is most inconvenient. It is the object of this note to describe improvements which make the dilution colorimeter as rapid and simple to manipulate as any other. These improvements partly concern the colorimeter itself, and partly the source of light employed.

Source of light for colorimetric purposes. The only satisfactory natural source of light for colorimetric purposes is the diffused daylight of a northern sky, but even if this be available its varying intensity is a drawback, because, for any coloured solution, there is a particular intensity of coloration at which a colorimetric comparison is most exact. An unvarying artificial source is therefore preferable.

There is now obtainable under the name of the Sheringham Daylight Lamp a source of light which is identical in quality with the diffused daylight of a northern sky, and does not vary in intensity. Such operations as comparisons in Nessler cylinders can be done with greater ease by this light than by genuine daylight for this reason, and it forms an admirable illuminator for any colour matching instrument.

In this lamp the light from a metallic filament lamp is reflected on to an inverted bowl which is pigmented over strictly proportioned areas with three different pigments, of which a blue predominates. The resulting illumination on a white paper a few inches away is sensibly uniform, and as there is no direct illumination the trouble of actual images does not arise.

Improved dilution colorimeter. The dilution colorimeter in its original form [Stanford, 1913] has the usual pair of rhomboidal prisms, each of which illuminates one-half of the field of view in the eye-piece. In front of the prisms there is a box containing two parallel sided glass cells, one of which

contains the standard solution, and the other the unknown solution. Over each cell is a burette, so that solvent can be run into the stronger solution until equality of colour is reached. The practical disadvantage of the instrument is the eye strain and waste of time involved in constantly withdrawing the eye from the eye-piece and the hand from the stirrer for the purpose of adding cautiously a drop or two more of solvent. The attachment to be described removes this inconvenience. It is merely necessary to pull a string, and drops of solvent issue from the burette as long as the string is pulled and at a rate previously determined by the observer.

Description. The colorimeter as shown in the accompanying drawings (Figs. 1 and 2, which are to scale) consists of two parts, namely, the light box, *ABD*, and the colorimeter proper.

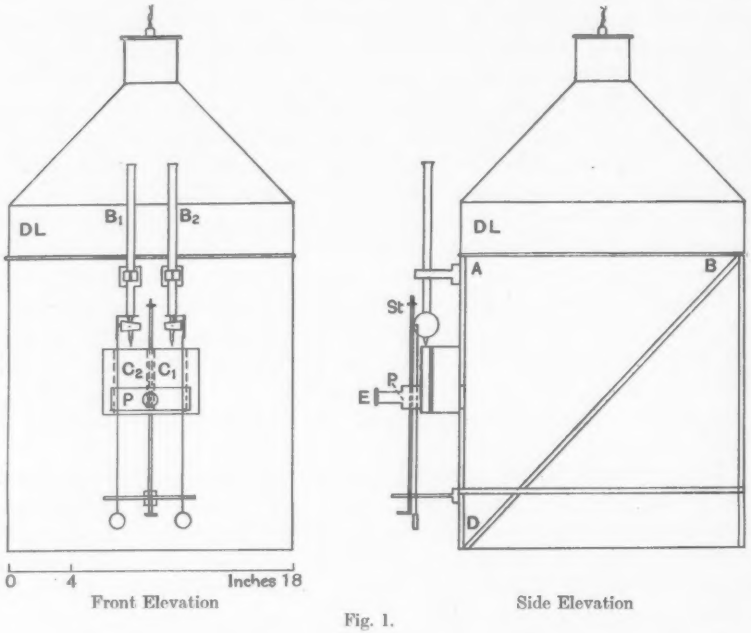

Fig. 1.

The light box consists of a cubical wooden box, each side of which measures 18″, bisected diagonally by another piece of three-ply, shown as *BD* in the side-elevation. On the top of the box is a Sheringham Daylight Lamp, *DL*. The particular lamp required is known as type 830*A*, and has a conical reflector 18″ in diameter.

The semi-cubical space, *ABD*, is lined throughout internally with white drawing paper, and in the front wall, *AD*, has a horizontal slot centrally situated, and measuring 5″ by 1½″.

The colorimeter proper consists of a brass box to hold the two cells C_1 and C_2. This box is screwed to the front of the light box opposite the slot. To the front of this cell-chamber is affixed the case P containing a pair of rhomboidal prisms and the eye-piece E. The stirrup-shaped stirrer St (shown with the stirrup removed) passes through the centre of the prism case. It is spring-loaded, and is worked up and down by means of the finger.

Above each cell is a burette, held in a clip so that it can be easily swung to one side to facilitate removal of the glass cell. The special feature of these burettes is the automatic attachment to the tap which enables them to deliver drops of solvent into the cells at any pre-determined rate. This attachment is shown in side and front elevation in the drawing (Fig. 2). A metal plate C is fixed to the stem of the burette below the tap. This acts as a stop. The burette-tap is firmly gripped by two pieces of wood which are centrally attached to a metal disc (the lid of a small tin is convenient) about $2\frac{1}{2}''$ in diameter. Near its edge this disc carries the studs a, d and e, which are panel-pins held in position with spots of solder, and also the set-pin b. The stud d is attached by means of a spring or rubber band to any convenient anchorage. The stud a carries a thread terminating in a ring large enough to admit the finger. The device acts as follows. Normally, the tension of the spring attached to d brings the stud e against the burette, and in this position the tap is closed.

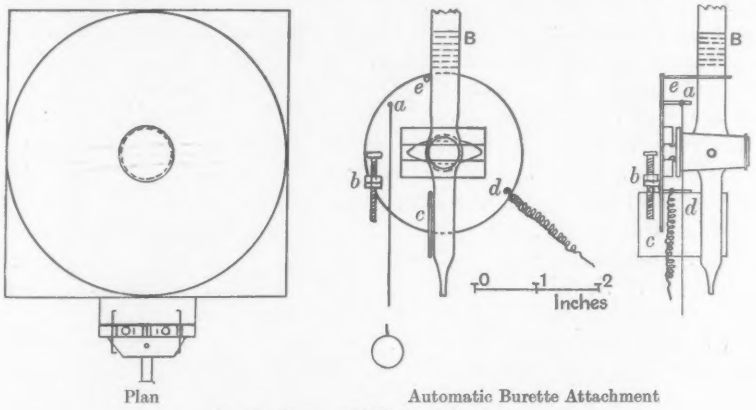

Fig. 2. Improved Dilution Colorimeter.

If now the thread attached to a be pulled down, the tap will open until the end of the set-pin b impinges upon the stop-plate c. By suitably adjusting the set-pin, the rate of drops from the end of the burette can be arranged beforehand. A rate of about one in some three seconds will be found convenient.

In using the instrument all that is necessary, therefore, is to place the two cells containing the liquids in position, switch on the daylight lamp and, while continuously working the stirrer with a finger of one hand, to pull the

string of the burette over the stronger solution, and allow the dilution to proceed until equality of colour is reached. The difference in the readings of the burette gives the volume of solvent added to the stronger solution, and the necessary simple calculation may be made with the help of the formula given below.

Calculation. Let

Total volume of unknown solution be V_u.
Amount of substance contained therein be x.
Volume of solution placed in colorimeter cell be v_u.
Total volume of standard solution be V_s.
Amount of substance contained therein be y.
Volume of solution placed in colorimeter cell be v_s.
Volume of water added in dilution be W.

Then

A. *When standard is diluted:*

$$x = \frac{V_u \times v_s \times y}{V_s \times (v_s + W)}.$$

B. *When unknown is diluted:*

$$x = \frac{V_u \times (v_u + W) \times y}{v_u \times V_s}.$$

Degree of accuracy of the instrument. Some months' use of the instrument has shown that the time required for a colorimetric comparison is about three minutes for any person of average perception in regard to colour.

Apart from this, under the heading of degree of accuracy, the same two points must be dealt with as in all other colorimetric devices, namely, what sources of error may be inherent in the instrument (whether from its principles or from its construction) and what is the degree of accuracy of the actual adjustment of the instrument when equality is reached.

As regards errors due to principles, this colorimeter has none. When equality is reached the liquids under comparison are of the same concentration, they are being viewed through the same thicknesses of layer, and therefore, like Euclid's triangles, they must be equal in all respects.

Under errors of construction come (1) unequal illumination, (2) inequality of the cells, and (3) lack of symmetry of the optical arrangements. Now as to these, the daylight lamp arrangement described entirely obviates unevenness of illumination, which is a fruitful source of trouble in colorimetry as a rule. The internal thicknesses of the cells are tested beforehand by dusting them with lycopodium powder and focussing a measuring microscope first on one surface and then on the other. The two cells should not differ by more than 0·1 %. The symmetry of the optical arrangements is controlled by examining in the colorimeter a number of liquids of different colours, a portion of each being put into each cell. In this connection it may be remarked that the only trouble likely to be met with is an unsymmetrical mounting, or a subsequent displacement, of the rhomboidal prisms.

As a result of a considerable number of experiments, it may be stated that a person with an average eye may expect to get results correct to 1 %, provided the comparisons are undertaken at the most sensitive depth of colour for the particular liquids under examination. [Compare Horn, 1906; Horn and Blake, 1906.] This refers to rapid and solitary estimations. If circumstances permit, say, three independent comparisons to be made, the error of the mean will be considerably less than this.

Mechanical stirrer. The simple form of hand stirrer described in connection with the colorimeter answers very well when only a few estimations have to be made, but becomes tedious and fatiguing if the instrument is in constant use. For this reason a mechanical stirrer is an advantage. It is clear that such a stirrer must be instantly detachable, for both it and the cell in which it works have to be cleaned and dried after each estimation.

Our stirrer is made from a dentist's drilling engine, which consists of a flexible shaft, passing through a flexible cable and carrying at one end a pulley and at the other end a spring bayonet arrangement by means of which the shaft can be instantly connected with the so-called "hand piece." This hand piece contains a small chuck for holding the drills. The actual stirrer is a piece of $\frac{3}{16}''$ glass rod flattened and twisted at the lower end to form a kind of screw, and mounted at its upper end in a socket soldered to an ordinary dental drill. The hand piece is rigidly fixed on a T-shaped piece of wood which slides in two wooden grooves screwed to the front of the light box. When the cells are in place, the piece of wood carrying the handpiece and stirrer is dropped into its slots, the end of the cable pushed on to the bayonet joint, and everything is ready for the estimation. The stirrer is driven by any convenient small motor.

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CIII. NESSLERISATION, AND THE AVOIDANCE OF TURBIDITY IN NESSLERISED SOLUTIONS.

By ROBERT VINER STANFORD.

From the Chemical Laboratory, Cardiff City Mental Hospital.

(*Received September 20th, 1923.*)

The brown coloration which is produced when very dilute solutions of ammonium salts are treated with Nessler solution has been used as a means of detecting and estimating ammonia in water analysis for many years, and more recently has found extensive employment in biochemical investigations, where the quantity of material available so frequently necessitates colorimetric analytical methods.

The quantities of substance which are measured in this kind of work are most conveniently reckoned in hundredths of a milligram. To avoid the constant repetition of this clumsy phrase, I venture to coin the word "centimilligram," conveniently written cmg.

In the case of every comparison of coloured substances there is a particular concentration at which the comparison is most easily made. This will depend, of course, on the thickness of the layer of liquid which is to be examined, and it varies with different colours, and to some extent with different individuals. Our comparisons are conducted in the dilution colorimeter [Stanford, 1923, 1] using a 10 mm. layer. In these circumstances the most satisfactory results are obtained when the concentration is in the neighbourhood of 1 cmg. of nitrogen per cc.

Nesslerisation differs also, however, from the other common colorimetric processes in that it is peculiarly liable to give rise to solutions which are *turbid*, and so useless for colorimetric comparison. This is well known, but until now its cause does not seem to have been discovered. It is stated in Clowes and Coleman's well-known handbook that the Nessler solution must always be added to the ammoniacal solution, and never in the reverse order, or a turbid solution will result. As will be shown, this statement is not correct. In the past few years Folin has described several rapid methods for the estimation of nitrogen or urea nitrogen in products of animal origin "by direct nesslerisation," that is to say, the reaction product is treated with Nessler solution and compared with a nesslerised standard. Cole [1920] has stated that he was unable to repeat these experiments because of the turbidity of the resulting solutions. The trouble seems also to have occurred to Folin himself, for he says, in a later paper, "if the solution be turbid, centrifugalise

a portion of it." A strange counsel, seeing that the turbidity is due to minute oily drops of the dark brown colouring matter which it is proposed to estimate.

After very numerous repetitions of Folin's procedure I could only confirm Cole's statement, but in further investigating the matter I was able to discover the cause of the occurrence of turbidity, and the means of avoiding it. The question really divides itself into two. On the one hand there have to be considered the processes which occur when a pure solution of ammonium sulphate or ammonium chloride is treated with Nessler solution, and on the other hand what occurs when, say, the product of a urine digestion is treated with Nessler reagent.

I have not been able to discover any conditions in which such impure products may be nesslerised to give a clear solution, and it is for that reason that I devised the method for the removal of ammonia from such liquids which is described in the following paper [Stanford, 1923, 2]. Some points with regard to such solutions will be referred to below.

Method of nesslerisation to obtain clear solutions. A clear solution will always be obtained (1) if the concentration of ammonia is below a certain limit (see below) and (2) if the addition of the Nessler solution be made *drop by drop with constant shaking.*

It does not matter whether the reagent is added to the ammonia or *vice versa* provided the addition be carried out in this way, but if the ammonia solution be added to the reagent, the latter should be diluted if possible beforehand.

It ought to be mentioned that two Nessler solutions are in common use, one of which is about twice as concentrated as the other. All the statements in this paper refer to the more dilute solution (about 22 g. KI per litre), but they hold good also for the stronger solution.

Ammoniacal solutions to be nesslerised should not contain more than 2 or 3 cmg. of nitrogen per cc. Turbidity can be avoided when the solutions are a good deal stronger than this, but it is not desirable to try, for a concentration of 1 cmg. of nitrogen per cc. gives a coloration convenient for colorimetric comparison in a 10 mm. layer.

The following experiments demonstrate the whole matter quite clearly:

(1) A solution containing 1 cmg. N per cc. (as $(NH_4)_2SO_4$) nesslerises clear when treated with one-tenth of its volume of Nessler solution added drop by drop with constant shaking.

(2) Repeat No. 1 without shaking and a turbid solution results.

(3) Repeat No. 1. The clear solution when treated with a few drops of 20 % sodium hydroxide becomes turbid at once, and the same thing happens if an excess of Nessler reagent be added.

(4) Repeat No. 1, but add to the ammonium sulphate solution a little urease solution (Folin) first; the nesslerised liquid will be turbid even though the reagent be added in drops and with shaking.

Ageing of nesslerised solutions. It is well known that the colour of a nesslerised solution alters rather rapidly with lapse of time. It was hoped that when the solutions were obtained with the precautions above mentioned this alteration might not occur, but unfortunately this is not the case. It is, therefore, still necessary to nesslerise solutions intended for comparison as nearly simultaneously as possible.

SUMMARY.

(1) Pure solutions of ammonium salts always give clear solutions when the Nessler reagent is added drop by drop with constant shaking.

(2) The ammonia solution should not contain more than 3 cmg. of nitrogen per cc., and the Nessler reagent should be one-tenth of the volume of the solution to be nesslerised.

(3) Excess of alkali or of Nessler reagent makes the clear solution turbid, as also does the addition of traces of urease.

(4) No conditions could be discovered in which clear solutions could be obtained by "direct nesslerisation" as suggested by Folin.

I wish to acknowledge my indebtedness to the Medical Research Council, by whom a part of the cost of this work has been defrayed, and also to Mr A. H. M. Wheatley for his cordial assistance.

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CIV. METHOD FOR THE RAPID AND QUANTITATIVE REMOVAL OF AMMONIA FROM SOLUTIONS, ESPECIALLY APPLICABLE TO THE MICROQUANTITATIVE ESTIMATION OF NITROGEN AND UREA IN PRODUCTS OF LIVING ORIGIN.

By ROBERT VINER STANFORD.

From the Chemical Laboratory, Cardiff City Mental Hospital.

(*Received September 20th, 1923.*)

Many analyses of biochemical importance end in the estimation of ammonia. The Kjeldahl process, in one or other of its countless modifications, is everywhere in use, and of recent years the estimation of urea by means of the Soya bean, or of the urease extracted from it, has become almost as much a matter of course.

All these processes leave the nitrogen in the form of an ammonium salt mixed with many other things. Several methods are in use to bring the ammonia into a pure solution in which it can be estimated. The mixture is generally made alkaline, and distilled, or alcohol is first added, or it may be distilled in steam. Another variation is the "aeration method" (drawing a vigorous current of air through it) as proposed by Folin and adopted by van Slyke.

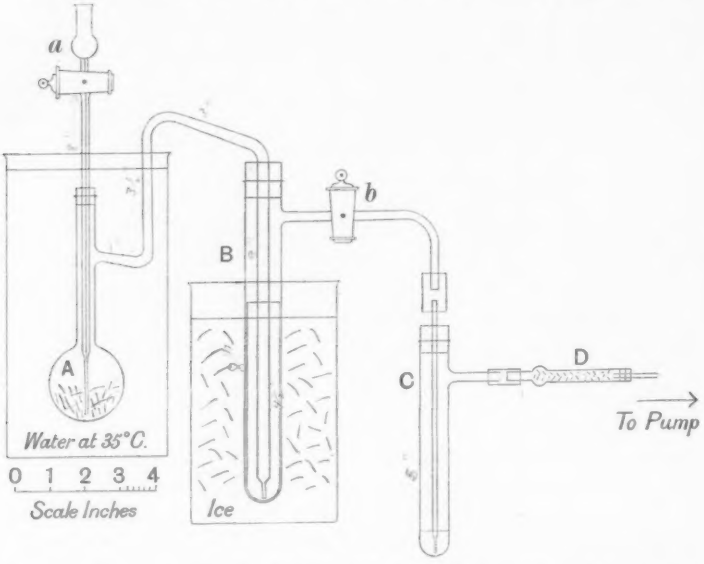
Folin has recently [Folin and Youngburg, 1918] sought a way out of the difficulty by estimating the ammonia by "direct nesslerisation." Unfortunately, owing to the presence of other colloids this nearly always gives turbid solutions (compare preceding paper) which are useless for colorimetric comparison.

The distillation and aeration methods are quite satisfactory as long as the quantity of ammonia to be measured is within volumetric limits, but in biochemical work the quantities to be measured are often so small that volumetric procedure would be absurd. In the case of the total nitrogen, non-protein nitrogen and urea nitrogen of cerebrospinal fluid or of blood, for example, microquantitative methods (in this instance colorimetric) are unavoidable, and it is therefore of importance to have some quick and quantitative means of removing the ammonia from the reaction mixture and getting it in the form of a pure solution which on nesslerisation will give a clear liquid for the colorimeter.

The aeration method is not applicable, for the slight losses which are bound to occur, and which are negligible in, say, a urine analysis, become comparable with the whole quantity of ammonia to be estimated—a quantity which may not exceed a few hundredths of a milligram.

Distillation may be employed, as in my method for the estimation of the total nitrogen of cerebrospinal fluid [Stanford, 1913], but would not be

suitable for urea estimations owing to the decomposition of the urease by the boiling alkali. The distillation method as there described has the advantages that it is easily carried out (except for the actual colorimetric comparison) by unskilled hands and it does not require any special apparatus. On the other hand it consumes a good deal of time, and this is inconvenient if a large number of estimations has to be made. For this reason we now do all our nitrogen estimations with the vacuum apparatus about to be described.



Vacuum Apparatus for the Distillation of Small Quantities of Ammonia.

The apparatus takes the form shown in the accompanying drawing. *A* is a small (100 cc.) distilling flask the side tube of which is lengthened and bent as shown, the end being drawn off to a fine jet. It is connected with the receiver *B* by means of a good rubber cork. In the receiver *B* is placed a thin, rimless test-tube of such diameter as to be an easy fit: this test-tube we term the container. *B* is connected through a tap *b* with the traps *C* and *D* and the pump. The distilling vessel *A* is provided with a capillary which carries a glass tap *a* and above that a small bulb.

The apparatus is used as follows. The "container" is removed from *B*, a wisp of glass wool is put in the bottom of it and the whole is then weighed to the nearest centigram. 4 cc. of very dilute (about $N/20$) sulphuric acid are run in, and the container is then replaced in *B*. The trap *C* is provided with about 3 cc. of dilute sulphuric acid and the tube *D* is filled with glass wool moistened with sulphuric acid. The receiver *B* is immersed in a beaker of ice and water containing enough water to enable the contents of *B* to be observed through it.

The liquid under examination is introduced into the distilling vessel *A*, together with a wisp of glass wool. If the solution is not faintly acid it must be brought to that condition, a scrap of litmus paper being thrown in to act as indicator. It is convenient so to arrange concentrations that the volume of solution taken shall be about 5 cc. The whole apparatus is now connected up as shown in the diagram, the mouth of *B* being previously smeared with glycerol, and, the tap *a* being closed, about 3 cc. of strong (5 *N*) sodium hydroxide solution are placed in the bulb above it. The distilling vessel is now surrounded with a beaker of warm water so that the cork at the top of the flask is completely in the water. The pump is turned on and the apparatus exhausted as completely as possible. With a good water pump this takes less than a minute. The most suitable temperature of the water in the bath will depend of course on the degree of vacuum obtained. A good pump should yield a vacuum of 12–13 mm., and in that case the warm water should be at 35°.

As soon as only an occasional bubble passes through *D*, the tap *b* is closed, and the tap *a* is opened to admit nearly all the sodium hydroxide solution. The apparatus is now left alone for five minutes, care being taken to maintain the temperature of the water-bath by placing a small flame underneath it. A continuous succession of bubbles will be seen being absorbed in *B*. The apparatus is functioning like the old cryophorus. During this period most of the ammonia passes over, and as the distillation is proceeding in a closed space there is no possibility of loss. At the end of five minutes connection with the pump is re-established by gradually opening *b*. Since the cork in *A* is sealed by immersion in water, and the cork in *B* by means of glycerol, no leaks can occur, but the vacuum is generally slightly impaired by reason of dissolved gases in the alkali solution which has been added. When connection with the pump has been completely re-established a fairly vigorous stream of bubbles will be seen passing through *B*, and an occasional bubble through the trap *C*. These are bubbles of water vapour which are only partially condensed in *B*, but they lose there any ammonia they may contain. The distillation is allowed to proceed in this manner for five minutes, counting from the time when the tap *b* first began to be opened. The tap *b* remaining open, the tap *a* is cautiously opened sufficiently to allow the slowest possible stream of air bubbles to pass through the distilling vessel, and this is allowed to continue for five minutes. The tap *b* is then closed, air is admitted through *a* and the apparatus is disconnected generally. The container is removed from *B* and weighed. In this way the total volume of the distillate is known, and it is then nesslerised with 1/10 of its volume of Nessler solution and compared with a standard in the dilution colorimeter.

It is obvious that the whole of the ammonia must be in this distillate unless any has remained in *A* or has passed over into the trap *C*. To check these possibilities, the liquid remaining in *A* is rinsed out into a Nessler cylinder, made up to 50 cc. and nesslerised. If the operation has been properly conducted not more than a barely perceptible coloration will be

observed. The contents of the trap *C* may then be poured in, and should cause no coloration either.

Summary of Procedure. The following summary of the procedure just described in detail should be intelligible on reference to the drawing.

(1) Exhaust the apparatus.

(2) Close *b* and admit sodium hydroxide solution through *a*.

(3) Both *a* and *b* remaining closed, allow the distillation to proceed for five minutes.

(4) Cautiously re-open *b* and let the distillation proceed a further five minutes.

(5) Admit the least possible stream of air through *a*, and continue for five minutes.

(6) Close *b*, admit air generally, re-weigh the container and nesslerise its contents.

(7) Nesslerise the liquid remaining in *A* and the acid in the trap *C* to check the completeness of the distillation.

Conclusion. Although, like most analytical schemes, rather lengthy in description the process is very quick and simple to carry out, for the distillation takes only 15 minutes, and during most of this time looks after itself.

The transference of the ammonia is almost strictly quantitative, for if any perceptible coloration is shown when the contents of *A* and *C* are nesslerised it does not exceed that due to one or two thousandths of a milligram of ammonia. It is true that even so small an amount makes a regrettable *percentage* error when only a few hundredths of a milligram of substance are there to be estimated, but percentage errors which could not be tolerated in ordinary analytical practice are inseparable from microchemical methods. Consider the experimental errors of the final colorimetric comparison, for instance.

It need hardly be pointed out that a blank experiment must be done to test the freedom from ammonia of any reagents that may have been used in arriving at the solution taken for distillation. Extensive use of the method has been made in this laboratory in connection with estimations of total nitrogen and urea in cerebrospinal fluid and in blood. In the case of the materials we have we find that blank experiments show the presence of less than half a hundredth of a milligram of nitrogen in the quantities of reagents used in any particular analysis, and as this is to a great extent off-set by the traces of ammonia remaining undistilled or passing through to the trap we do not take either error into calculation. Where reagents of a lesser degree of purity are met with the correction must of course be made.

I wish to acknowledge my indebtedness to the Medical Research Council, by whom a part of the cost of this work has been defrayed, and also to Mr A. H. M. Wheatley for his cordial assistance.

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CV. THE AZINE AND AZONIUM COMPOUNDS OF THE PROTEOLYTIC ENZYMES. I.

By HEDLEY R. MARSTON.

From the Darling Laboratories, University of Adelaide, South Australia.

(*Received September 24th, 1923.*)

The immense importance of the establishment of the chemical nature of the proteolytic enzymes has led to many endeavours to purify them. From the earliest attempts of Schwann [1836] to the present day all these have met with obvious failure.

In 1905 it was observed by T. Brailsford Robertson [1907] that if one drop of saturated safranine (Grübler) is added to a solution of trypsin (Grübler) a precipitate forms which flocculates in the course of a few hours and collects at the bottom of the tube. Some years later Holzberg [1913] demonstrated that the precipitate was proteolytically active. With these two facts in view a study of the nature of this precipitate was undertaken.

Preparation of the Safranine Precipitate.

To prepare a sample of the precipitate, 100 g. of commercial pancreatin (Armour) was extracted with 500 cc. of water and the insoluble fraction filtered off. The amber-coloured solution so obtained was treated with an equal volume of 0·5 % safranine (Grübler) and the resulting precipitate allowed to flocculate for half an hour and then separated by centrifugalisation at high speed for twenty minutes. After washing the resulting fine slimy sediment with 0·5 % safranine, excess of the dye and the water were removed by six successive washings with absolute alcohol. The precipitate was then washed with ether and dried at 40°. The yield constitutes about 2 % of the original pancreatin employed. The precipitate is a red, finely grained powder, which in the presence of water emulsifies to form a violet solution which shows a remarkably strong proteolytic activity when the reaction of the solution is adjusted to p_H 8·0. The compound is hydrolysed by dilute acid (0·2 % HCl) with liberation of the free safranine chloride which may be extracted from its solution by butyl alcohol. The treatment with acid, however, greatly impairs the proteolytic activity owing to inactivation of the dissociated trypsin.

The solution from which the safranine precipitate is removed is completely inactive.

A comparison of the activity of the precipitate with that of the solution from which it was removed, and of the original solution from which it was prepared, is shown by the following experiment.

Comparison of the Relative Digestive Activity towards Caseinogen of the Safranine-Trypsin compound and its Equivalent of Unpurified Trypsin.

Solution A. The trypsin employed was the partially purified and very active product prepared by Grübler in Leipzig and sold as trypsin puriss. sicc. A 2 % solution in distilled water was used, the small insoluble residue being separated by centrifugalisation. The solution being freshly prepared before each experiment was preserved in ice until wanted.

Solution B. 4 cc. of Solution A were treated with 4 cc. of safranine 0·5 % in a small centrifuge tube with a conical bottom, and after allowing to flocculate for thirty minutes the resulting precipitate was separated in a high-speed centrifuge. The supernatant fluid was decanted and preserved as Solution C. The sediment was washed twice on the centrifuge with 8 cc. of 0·5 % safranine and then six times with absolute alcohol brought to p_H 6·0 with alcoholic HCl. It was then taken up in 4 cc. $M/15$ phosphate buffer (p_H 7·5). 2 cc. of this solution contain the safranine precipitate from 2 cc. of Solution A.

Solution C. This solution is the residue from the precipitation of the azine compound, 4 cc. being equivalent to 2 cc. of Solution A.

The Substrate. Carefully purified caseinogen, prepared by a modification of the method of Van Slyke and Bosworth [1913], was dissolved, with the aid of a mechanical stirrer, in 80×10^{-5} g. equivalents of NaOH (*i.e.* 8 cc. $N/10$ NaOH per g. caseinogen). To this solution, which is neutral to phenolphthalein (p_H less than 8·0), was added 3×10^{-5} g. equiv. of HCl, the addition of the acid being slow and accompanied by rapid stirring. The solution was then diluted so as to contain 4 g. of caseinogen per 100 cc. This solution was diluted with an equal bulk of $M/5$ phosphate buffer solution, prepared by adding 166 cc. $M/5$ Na_2HPO_4 to 34 cc. $M/5$ NaH_2PO_4, the p_H of which is 7·5. This substrate was preserved by the addition of toluene (0·5 cc.).

Technique of Experiment. 100 cc. portions of the substrate were measured into four long-necked glass-stoppered glass flasks and immersed in a thermostat which was capable of regulating the temperature to within 0·01°. When they had reached the temperature of the bath equivalent portions (2 cc.) of each of the enzyme solutions A, B, C were added to the flasks from which, after shaking, 2 cc. portions were withdrawn by means of a dry pipette and delivered into 2 cc. of $M/6$ CH_3COOH. Subsequently 2 cc. were withdrawn from each at half-hourly intervals and treated similarly. After allowing to flocculate the caseinogen was filtered off and the refractive index of the clear filtrate was determined by means of a Pulfrich refractometer, reading accurately to within 1 minute of the total angle of refraction, the refractive index of the

prism being 1·62098 (experimental error 0·00078 of the refractive index). The amount of caseinogen was determined from the formula:

$$\text{Caseinogen } \% = \frac{2(n-n')}{0{\cdot}00152},$$

where n is the refractive index of filtrate,

n' is the refractive index of control (prepared at 0 hour's digestion period),

0·00152 is the change of refractive index due to 1 g. of digested caseinogen in 100 cc. of solvent [Robertson, 1912].

The refractive index method of determining the rate of protein hydrolysis has the objection that the experimental error is large, being ± 0·1 g. of the caseinogen in the digest. However, with this difficulty in view, the velocity constant calculated from the mono-molecular reaction was found to be constant within the limits of the experimental error. The results are shown in Table I.

Table I.

Substrate = caseinogen 2 %. p_H = 7·5. Refractive index = 1·33743. Temperature 40°.

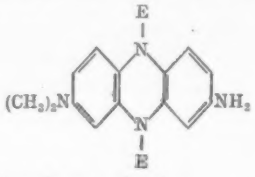
	Time (t) hrs.	Angle of refraction	Refractive index 0 hrs. = n_1	Refractive index at (t) = n	G. digested $= \frac{2(n-n_1)}{\cdot 00152}$	a	x	$K = \frac{1}{t}\log_{10}\frac{a}{a-x}$
Enzyme solution A, 2 cc. (original solution)	0	66° 39′	1·33591	1·33591	0·0	—	—	—
	½	66 36	1·33591	1·33614	0·3	2	0·3	14×10^{-2}
	1	66 33	1·33591	1·33639	0·5	2	0·5	12
	1½	66 31	1·33591	1·33655	0·8	2	0·8	14
	2	66 29	1·33591	1·33671	1·0	2	1·0	15
Enzyme solution B (azine precipitate from 2 cc. of A)	0	66 39	1·33591	—	0·0	—	—	—
	½	66 37	1·33591	1·33606	0·2	2	0·2	9
	1	66 35	1·33591	1·33623	0·4	2	0·4	9
	1½	66 32	1·33591	1·33647	0·6	2	0·6	10
	2	66 31	1·33591	1·33655	0·7	2	0·7	9
Enzyme solution C (residue from azine precipitate B)	0	66 39	1·33591	1·33591	0·0	—	—	—
	2	66 39	1·33591	1·33591	0·0	— inactive		—

The table explains itself. The azine precipitate which constitutes only approximately 2 % of the preparation has retained at least 70 % of the activity shown by the original enzyme solution. The loss may be due to partial inactivation of the enzyme during the rather rough treatment it receives, but, in all probability it is due to the incomplete dissociation of the enzyme-azine compound. The solution from which the azine precipitate was removed was completely inactive.

With the possibility in view of determining the nature of the linkage between the enzyme and the safranine molecule, the action of the other azine bases (eurhodines, safranines, indulines) towards the proteolytic enzyme trypsin was determined. Of the series tried, all those compounds which were water-soluble and contained the azine nucleus were capable of completely precipitating the enzyme from its solution.

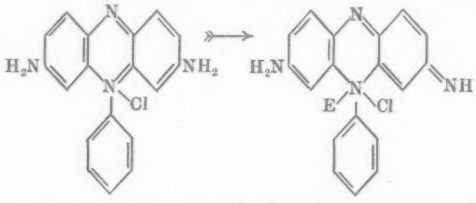
Thus dimethyldiaminophenazine hydrochloride (neutral violet), dimethyldiaminotoluazine hydrochloride (neutral red), diaminophenyltoluazonium

chloride (safranine), dimethyldiaminophenylphenazonium chloride, all quantitatively precipitate the active principle from an impure trypsin solution. Even the complex aminodianilinophenylphenazonium chloride (indamine blue) is active in the precipitation. The type of union between the enzyme and azine base proved to be a direct combination with the azine nitrogen. In the case of dimethyldiaminotoluazine hydrochloride the union would be represented thus, where E represents the Enzyme molecule:



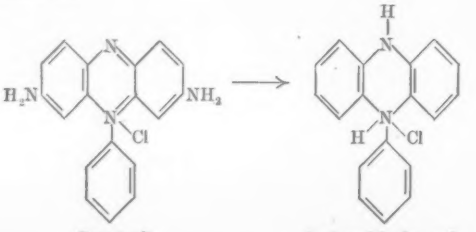
or in the case of the azonium base, safranine

Safranine (red) Safranine-enzyme cpd. (reddish violet)



The mono-enzyme compound would alter the constitution from the ortho- to para-quinoid structure, with a consequent tautomeric rearrangement and accompanying colour change. The colour change, from red to violet, is noticeable in the precipitate when redissolved. This may possibly be due to the occurrence of such a tautomer. The analogous mono-acid salt of safranine, violet in colour, occurs in solutions of moderately high acidity.

Confirmatory evidence of this hypothesis is available. The above reactions would necessitate the enzyme or combining substance to be predominantly acid in character. That this is so, is inferred from the experiment of Bayliss [1906] who showed that trypsin migrated to the anode when subjected to the influence of an electric current. This would be expected if the trypsin were acidic in character. The complete loss of the ability to precipitate the enzyme by the reduced "leuko" compound further supports the hypothesis of its linkage to the azine N.



Dye (red) Reduced leuko cpd

The enzyme or the substance which carries down the enzyme so completely is precipitable by the azine and azonium bases, the basic N of the azine nucleus being active in this precipitation.

The study of the action of the azine bases was extended to other enzyme preparations. By means of fractional precipitation with alcohol and subsequent dialysis, very active preparations of trypsin may be prepared from extracts of the pancreas. However, the proteolytically active substances, precipitated by the azine bases from these so-called "pure trypsin" preparations, constitute but a very small proportion of the whole material, rarely more than 5 %. No preparation was examined from which the proteolytically active substance could not be precipitated quantitatively by the azine bases.

The azine precipitates from crude gland extracts retain their proteolytic activity, but show no lipolytic or diastatic activity, although both of the enzymes responsible for the latter actions may be demonstrated with ease in the original glandular extracts. The experimental data of this work will be published in a later paper.

The substance precipitable by azine retains the power to clot milk. As this property is common to all the proteolytic enzymes, the action of the azine bases was tried towards proteolytic enzymes from other sources, with the remarkable consequence that in all cases the azine base completely precipitates the whole of the proteolytically active substances from their solutions, thus:

Enzyme	Source	Base	Activity of azine ppt.	p_H
Pepsin	Stomach extract (pig)	Pheno-safranine	+	2 (HCl)
Trypsin	Pancreas (pig)	,,	+	7·5
Erepsin	Succus entericus (pig)	,,	+	7·5
Papain	Paw-paw (Parke, Davis and Co.)	,,	+	5·0
Erepsin	Yeast	,,	+	7·5

Fibrin was used as substrate in the case of the pepsin and trypsins; "Difco" standard peptone being used for the demonstrations of the activity of the erepsins.

Separation of Pepsin from Azine-pepsin Compound.

Owing to the stability of pepsin towards acids the pepsin-azine compound was further studied. At p_H 1·2 the compound is decomposed, and the base may be extracted with amyl or butyl alcohol. When the compound is shaken in a separating funnel with 0·5 % HCl and butyl alcohol, and allowed to stand, three layers are formed. The butyl alcohol layer contains most of the azine. A stable emulsion of butyl alcohol constitutes the second layer. The third or watery layer which should contain the free enzymes, however, is inactivated by this process. The active constituent was found to be adsorbed at the surface of the butyl alcohol droplets in the emulsion. When this emulsion is broken up by the addition of a few drops of ether, the original proteolytic activity is regained.

The pepsin-azine precipitate has been prepared in quantity. It is not widely different in its physico-chemical behaviour, from the trypsin-azine

precipitate. However, it is soluble in 0·5 % HCl. The effect of the quantitative precipitation of the proteolytically active substance from solution by the azine bases may be easily demonstrated with pepsin solutions because of their relative stability.

Discussion.

The power of the azine and azonium bases to remove completely the proteolytic enzymes from solution indicates that a direct union of the enzyme and the precipitating base is established. The linkage has been shown to occur through the basic nitrogen of the heterocyclic azine ring; this union, instituting a tautomeric rearrangement within the azonium base, causes a colour change. The recovery of the proteolytic power of the azine-enzyme compound depends on the conditions which most favour its dissociation. The compound is more highly dissociated by an increase of H ions. At p_H 2·0 the pepsin-azine compound, for example, is decomposed and the red azine dye set free.

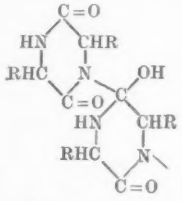
The compound is but slightly soluble in water at neutrality, but complete solution is obtained by the addition of peptone or protein digestion products. This, in all probability, is due to the dissociated enzyme ion combining with the protein digestion products, liberating the azine which reverts to the ortho-quinoid structure, the whole compound thus being decomposed and carried into solution.

The combination of the proteolytic enzyme with the azine nitrogen gives us evidence as to the nature of the combination between this enzyme and its substrate.

From time to time in biochemical literature the occurrence of diketo-piperazines in protein digests has been demonstrated. The presence of leucine anhydride among the cleavage products was first mentioned in 1849 by Bopp [1849]. Salaskin [1901] isolated diketo-piperazines from peptic and tryptic digestion products of oxyhaemoglobin. Abderhalden [1903] showed the presence of about 1 % of leucine anhydride in the products of HCl hydrolysis of caseinogen. He suggested that this anhydride was produced as a secondary product of leucine, and attempted, unsuccessfully, to demonstrate the reversal of the reaction. Water hydrolysis of proteins at high temperature produces about 1 % of leucine anhydride [Graves and Marshall, 1917].

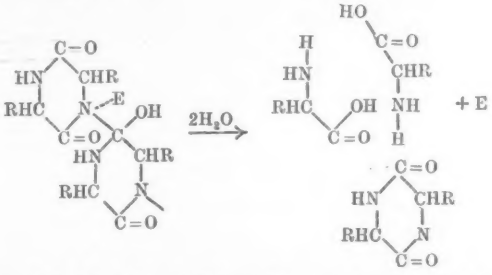
The catenary or chain-like configuration, as accepted in modern protein literature, fails to explain many peculiarities of the chemical actions of proteins. Such a structure would necessitate at least one free amino group at the end of the molecular complex. However, contrary to this, Van Slyke and Birchard [1914], and others, have shown that the nitrogen liberated from native proteins by the action of nitrous acid is equal to one-half of the lysine nitrogen content, that is, it is liberated from the NH_2 groups of the lysine bound up in the protein molecule. Some proteins, salmin and zein for example, when pure do not yield any nitrogen with nitrous acid, thus demonstrably lacking any free NH_2 groups.

These facts, together with other evidence hitherto unpublished, have led the author to put forward the hypothesis of a new form of protein structure. Instead of the conventional structure, the protein molecule may be considered to be built up of a series of amino-acid anhydrides, or, in other words, protein has essentially a poly-diketo-piperazine structure. Taking the type amino-acid $R.CH(NH_2).COOH$ for example, the protein complex is represented by this type of structure as



Such a structural configuration of the protein molecule would make quite clear the absence of terminal NH_2 groups. Peculiarities of protein ionisation and their neutralising power may be easily explained by means of this suggested structure. The similarity of each ring to the azine nucleus of the bases which are functional in combining with the enzyme suggests that the N in these groups is the seat of action of the enzyme.

The proteolytic enzyme E, combining with the nitrogen (N) of the diketo-piperazine ring would necessitate a structural change to supply the fifth valency of the nitrogen atom.

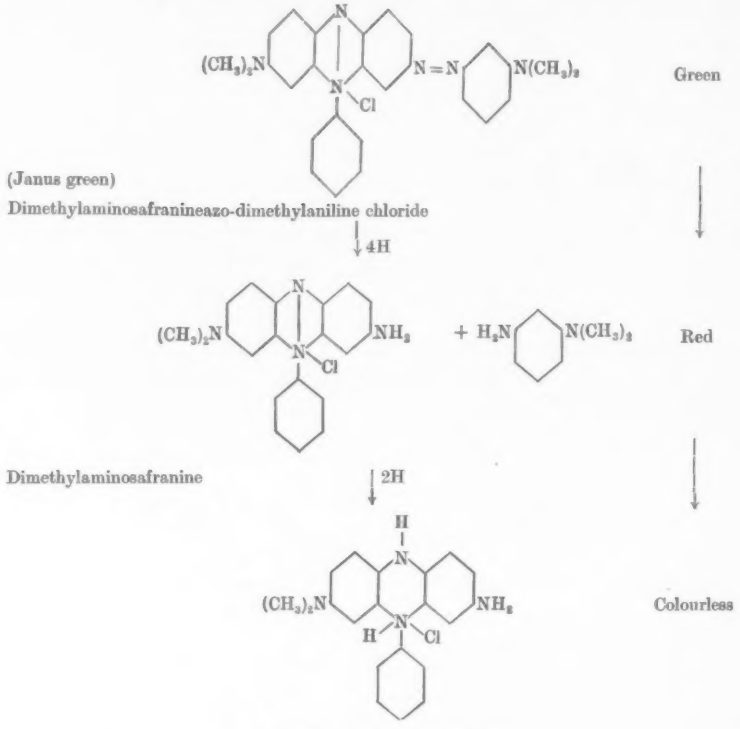


This could be satisfied by the addition of two molecules of water, resulting in the separation of two molecules of amino acid. The enzyme at this point is set free to act on a fresh group.

As the azine and azonium bases are coloured substances the occurrence of the proteolytic enzymes in the cell may be demonstrated by means of their selective staining reactions.

Michaelis, in 1899 [quoted by Cowdry, 1918, p. 86], while making a detailed study of the behaviour and chemical nature of the *intra vitam* dyes, found that dimethylsafranineazodimethylaniline (Janus green) specifically stained certain structures in the living cell. These structures have since been demonstrated

in all cells, from the bacterium to the angiosperm in the plant kingdom, and from amoeba to man in the animal kingdom. Cowdry [1918] showed that ethylsafranine will also stain specifically the mitochondria (as these structures are generally named). He also demonstrated that the preparation stained with Janus green, if kept under anaerobic conditions, will manifest a series of colour changes, the mitochondria changing from green to red and then becoming colourless. This, he points out, is due to the reduction of the dyestuff. If we follow out the reactions involved



leuko-Dimethylaminosafranine.

we see that the point of combination of the dye with the mitochondria could be no other than the N of the azine nucleus. After the stained tissue preparation is bleached by reduction it cannot be restained by application of more dye, so the groups to which the dye is linked are fully saturated with the dye. The modification of the mitochondrial constituents of the cells into zymogen granules during active secretion of the pancreas has been demonstrated by various workers.

The specific staining of the mitochondria with the azine dyestuffs indicates that the proteolytic enzymes are concentrated in these bodies. The mitochondria may be thus the site of syntheses in the cell, the water-poor phases which exist at the surface of the lipoid constituents of the mitochondria instituting favourable conditions for the synthetic activities of the enzyme.

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Note.—The possibility of the existence of diketopiperazine rings in the protein molecule is discussed by Abderhalden [*Z. physiol. Chem.* 1923, **128**, 119] in a paper which appeared in May 1923 but was not accessible in Adelaide at the date when Mr Marston's paper was despatched to England. EDITOR.]

CVI. SOME EXPERIMENTS AND REMARKS ON THE POSSIBLE TRANSFORMATION OF *d*-GLUCOSE IN THE INTESTINE AND ON THE NATURE OF BLOOD-SUGAR.

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In this article are communicated the results of experiments on carbohydrate-metabolism, which are closely connected with those recently published by a number of English investigators. These experiments deal, namely, with the possible transformation which the stereoisomeric glucose-forms, present in a solution of *d*-glucose in equilibrium, undergo in contact with the intestinal mucosa, and with the nature of blood-sugar.

I began these investigations at the suggestion of Prof. Hamburger as early as July, 1922. At first it was not my intention to communicate the results of these experiments yet, though their number in both problems was a large one. However, as I shall not have opportunity to continue my experimental work on these and allied subjects in the nearest future, I give here a short account of my work, especially as I am informed that the publication of the results reached up to the present may be of value for others, and also, as in more than one respect the methods used are different from those followed by the English investigators.

The reason for undertaking the experiments on the subjects mentioned, was the results of the investigations of Prof. Hamburger and Dr Brinkman on the permeability of the kidney towards isomeric and stereoisomeric sugars, which were published in different papers [Hamburger, 1919, 1, 2; 1921, 2; 1922, 1; Hamburger and Brinkman, 1918, 1], and discussed by Prof. Hamburger in two lectures [Hamburger, 1921, 1; 1922, 2]. The results of these investigations seemed to prove that the permeability of the kidneys for the stereoisomeric forms of the same sugar could be quite different.

Of these numerous experiments I wish to draw attention here only to those concerning the partial retention of the kidney for *d*-galactose [Hamburger, 1921, 1, 2; 1922, 1, 2], which was always about 50 %. Therefore Hamburger concluded that the only reason for this partial retention could be the fact, that of the two modifications of *d*-galactose in solution, namely the α- and the β-variety, one form is retained by the kidney, the other form is not held back.

Similar results were obtained with the two varieties (α- and β-) of another mutarotatory sugar, *l*-xylose. Here 25 % was always retained.

To study the mechanism of this difference, a number of experiments were made. In addition the question was put whether the difference in permeability, found between the isomeric and stereoisomeric forms of the same sugar, might also have a practical significance for the diabetes problem. That means, the question was introduced by Hamburger, whether the quite different behaviour of the normal and diabetic organism towards glucose had as underlying principle a stereoisomeric difference between the glucose of the blood under normal and pathological conditions [Hamburger, 1922, 2].

In the literature we find a few examples of a different behaviour of the organism—of the normal as well as of the diabetic—towards the stereoisomeric forms of the same sugar. Thus a difference was found in the behaviour of the human body towards different stereoisomeric methylglucosides [Lit. cited by Neuberg, 1911]. But that really in the normal organism one special stereoisomeric form of glucose is preferred, seemed to be demonstrated by the well-known experiments of Hewitt and Pryde [1920]. They seemed to indicate with some certainty, that normally by the resorption of carbohydrates in the intestine these are not only all transformed into glucose, but principally into one definite stereoisomeric form of glucose of a very reactive nature and with a negative or weak positive specific rotation (γ-glucose?). They would also show us that we might expect the possibility of the presence of such a reactive sugar, which is in equilibrium with *d*-glucose, in the blood. It is well known to the readers of this *Journal*, that in the recent investigations of Winter and Smith [1922; 1923, 1, 2, 3, 4; also Forrest, Smith and Winter, 1923] the existence of such a reactive sugar in the normal blood is assumed to be very probable.

As both questions, the transformation of *d*-glucose in the intestine and the nature of the blood-sugar, were fundamental and might be closely connected, Prof. Hamburger advised me to take up the problem of the stereoisomeric sugars. At his suggestion a beginning had already been made with a repetition of the experiments of Hewitt and Pryde. I have continued and extended these experiments.

In the first series of experiments in our laboratory only narcotised rabbits were used and aqueous solutions of very pure glucose in equilibrium of a strength of 2 to 3 %.

As the results reached in these experiments wholly correspond to those just published in detail in this *Journal* by Stiven and Reid [1923], I may refrain from a detailed statement of our results. This especially as our technique, as regards filtration and care for accurate polarimetric readings, largely corresponded to that followed by these authors.

About the procedure I only wish to say, that we worked so quickly, that the first polarimetric readings of the clear filtrate were always made within three minutes after the liquid had been removed from the intestine. The polarimeter which we used, in these and the following experiments, was a two-field instrument with which readings to a maximal accuracy of 0·01° could be made. The readings in these experiments had in nearly all a maximal error of 0·04°. The solution remained in contact with the mucosa from 5–15 minutes.

For washing the loop of intestine before it was filled with glucose, we used in two experiments Tyrode's solution made up without glucose, and in four experiments a modified Ringer's solution of the following composition: NaCl 0·7, KCl 0·01, $NaHCO_3$ 0·2, $CaCl_2$. 6 aq. 0·02 %.

Before the beginning of the experiment we passed CO_2 through this solution till $[H^+]$ was $0{\cdot}45 \cdot 10^{-7}$ (determined with neutral red as indicator). In this way we had a solution of which the $[H^+]$ and $[Ca^{++}]$ correspond to those of the serum of the animal used, and which, from the well-known experiments on the retentive power of the kidney for sugar [Hamburger, 1919, 1, 2; Hamburger and Brinkman, 1918, 2], and from those on the resistance of the red corpuscles [summarised by Hamburger, 1922, 3], may be regarded closely to approach the normal physiological fluid.

I give here as illustration only the shortened report of one experiment.

Exp. A. Rabbit, weight 2500 g. No food was given in the last 24 hours before the experiment began.

Morphine-anaesthesia; 60 mg. HCl-morphine intravenous.

Glucose solution of about 2 %, prepared 18 hours before the beginning of the experiment; rotation in 20 cm. tube 2°·04 (mean of six determinations).

Beginning operation five minutes after morphine injection.

Intestine loop used, directly behind stomach, of about 40 cm. length, tied off, two canulae in the ends, washed out with Ringer fluid at body-temperature, then with glucose solution and filled with the latter carefully. Glucose in intestine from 10 h. 30–10 h. 44. At 10 h. 44 content of intestine loop filtered and filtrate investigated in 10 cm. tube.

10·45	+1·30°	10·54	+1·28°
10·48	1·32	11·07	1·26
10·49	1·26	11·21	1·26
10·51	1·29		

No alteration after addition of a trace of alkali.

It is seen, that in this case the first reading (in the table is always given the average of several readings) was made within one minute after the liquid had been removed from the intestine. The difference between the first and the following readings was in this and also in the other cases within the limits of permissible error.

Altogether six of these experiments with narcotised rabbits have been done, but like Stiven and Reid, who have done a much larger number of experiments, we have in no case obtained results of the same nature as those of Hewitt and Pryde.

In two of the six experiments we determined the reduction of the filtrate immediately and also after a few hours, without finding a difference of importance. Compared with the polarimetric value, the reduction, determined by Bang's method, was constantly a little higher.

We have repeated these experiments under conditions which in my opinion may be called more physiological than those with the narcotised rabbits; namely, we made use of a dog, in which I had made a *Vella-fistula* of the small intestine (the length of the intestine loop was about 40 cm.). With this dog I have repeated the experiments of Hewitt and Pryde, now of course without anaesthesia, at least 25 times, but, as may be said directly, also with negative result.

In these experiments the fistula was first washed out with the usual Ringer or Tyrode, heated to 37°. We then injected the 2 % solution of glucose in equilibrium, also heated to 37°, and at a certain moment, whilst one person injected the solution slowly into one end of the fistula, another closed the other opening with his fingers. Then the injection-canula was removed and the other opening also closed with the fingers. The dog was a very quiet one and with the openings of the fistula firmly closed with the fingers, remained standing for the whole length of time that the fluid remained in the intestine, *i.e.* for 5–10 minutes. Generally about 50–60 cc. of the glucose solution was in the intestine loop at the moment it was closed. At the end of the determined time we could not wait till a peristaltic action of the bowel removed the unresorbed liquid and therefore it was washed out with a quantity of the modified Ringer or

Tyrode. The liquid obtained was filtered directly and the clear filtrate investigated in the polarimeter tube at a temperature of 37°, although in the first four experiments we made the readings at room-temperature just as in the preceding experiments. The observation-time in the experiments with the dog was much longer than in those with the rabbits.

I give here as illustration a shortened report of one of these experiments, which may serve as a good example of the general results obtained in all. It is seen that also in this experiment we did not get results similar to those obtained by Hewitt and Pryde, though the conditions were, I believe, much more physiological.

Experiment 1 with fistula dog.

Glucose solution of about 2 % in equilibrium. Fistula loop washed out with Tyrode, made up without glucose.

3·21–3·29 glucose solution is in the fistula.

3·29 fistula is washed out with Tyrode.

Polarimetric readings in tube of 10 cm.

3·315	+0·74°	3·48	+0·70°
3·325	0·70	3·485	0·70
3·345	0·72	3·495	0·67
3·37	0·70	3·53	0·70
3·385	0·68	4·08	0·72
3·40	0·68	4·09	0·71
3·415	0·66	Next morning at 9.15	+0·68.
3·44	0·67	After addition of trace of alkali	no change.
3·45	0·68		

After we had read the experiments of Winter and Smith, which will be shortly referred to later on, we thought of the possibility, that *insulin* might play a rôle in the transformation of glucose in the normal intestine and that insulin was absent from the mucosa of the fistula loop. We have therefore, in four experiments with the fistula dog, added a trace of insulin to the glucose solution in equilibrium brought into the intestine, although our experiments in this direction with rabbits had also given negative results. However in these experiments we again did not get indications of mutarotation.

In the experiments with the dog we also very often compared the reduction and polarimetric value of the filtrate directly (within a few minutes after the clear filtrate had been obtained) and after one or two hours, once also after 24 hours. Here again we found no difference of importance.

Although I have often tried by altering the details in the various experiments (small variations of the p_H in the modified Ringer used, etc.) to get a positive result, that is to say, to observe that the liquid obtained from the intestine showed an upperwards tending mutarotation of importance; yet, as already mentioned, and to my regret, like Stiven and Reid, I was never able to manage it, let alone to get a negative reading such as Hewitt and Pryde reported they had obtained once.

In the meantime we had also commenced to study the problem of *the nature of the glucose in the circulating blood*, since dealt with fully by Winter and Smith [1922, 1923, 1–4; see also Forrest, Smith and Winter, 1923]. We expected from the beginning that if the blood-glucose consisted mainly of one stereoisomeric form of glucose and that if we tried to investigate this by using blood itself, there was reason to expect a rapid change to the α-β-equilibrium; as the

blood had to be deproteinised before the reduction or optical rotation could be determined, which takes time. Then, if the deproteinisation is done chemically, this in itself might have an influence and invalidate the experiment[1]. Looking for other possibilities, we thought of the *aqueous humour* and of the *serum ultrafiltrate*; the first of these had been previously used in researches of this laboratory several times [van Creveld, 1921; de Haan and van Creveld, 1, 2].

For the problem in question, the aqueous humour, which may be regarded as an ultrafiltrate or better as a vivo-dialysate of the blood-plasma, has several advantages: it is colourless, water-clear, does not clot (the primary liquid at least) and contains only a trace of protein. It is therefore possible to determine reduction and polarimetric value of the liquid of the eye-chamber for the first time directly after puncture. A drawback was of course that the amount of glucose in the normal chamber liquid is only small, smaller than in the blood-plasma [de Haan and van Creveld, 1921, 1, 2]. Notwithstanding this disadvantage we thought it to be of great importance to see in how far with the eye-chamber liquid, which was not treated in any way, indications about the stereochemical forms of glucose in the blood under normal and pathological conditions might be obtained. We made the puncture of the cornea in the same way as in former researches with aqueous humour, namely with a sharp-pointed glass capillary, and in the upper part of the cornea, after cocainisation with 2 % HCl-cocaine. From each rabbit's eye about 0·3 cc. may be obtained.

First we looked for a qualitative test of the presence of a definite stereoisomeric form of glucose. As such the *decolorisation of permanganate* may be regarded, as it may point to the existence of a reactive sugar. We compared the rate of decolorisation of a 1/100 *N* weak alkaline solution of potassium permanganate by the eye-chamber liquid at different times after the puncture.

In these experiments with normal animals we found that, whereas *the addition of the eye-chamber liquid immediately after the puncture caused a rapid and intensive decolorisation of the permanganate,* this became slower the longer the time that elapsed between the time of puncture and the moment of addition to the permanganate. Using a proportion of 2 cc. *N*/100 $KMnO_4$ to 0·2 cc. eye-chamber liquid I found that after 25 minutes to half an hour, the rapidity of decolorisation had decreased in a high degree and was then not much larger than that of a 0·5 % solution of glucose in equilibrium, used for comparison. There is a possibility that the rapid decolorisation of permanganate by the fresh eye-chamber liquid is due partly to the presence of auto-oxidisable substances. It is difficult, however, in my opinion, thus to explain the whole decolorisation. The relatively slow decrease of the power of decolorisation is in any case difficult to account for in this way and it is also of importance to mention at this point that the reduction and the refractive index of the eye-chamber liquid, determined at different moments during the first hour after the puncture, appeared not to decrease. The change in the behaviour towards permanganate might thus surely be in favour of the presence of a reactive sugar in the normal aqueous humour.

To find out in how far other findings were in accordance with this, we investigated the polarimetric value of the aqueous humour during the first hour

[1] Compare here also the remarks of Hewitt [1923].

after the puncture. Here we made use of the micropolarisation method as described by E. Fischer [1911]. This was necessary, as the results with the permanganate test seemed to us to indicate that if the eye-chamber liquid showed mutarotation, the changes might take place during the first half-hour after the puncture. We therefore worked as quickly as possible, using only a small quantity of liquid, obtained from one or two eyes. Waiting till we had collected sufficient liquid from several rabbits to fill a common polarimeter-tube would have taken too much time.

Making use of the figures obtained in a former research, concerning the average sugar-content of the normal aqueous humour of the rabbit, we calculated whether the possible mutarotation of this liquid could be determined with sufficient accuracy. We found that, accepting that the specific rotation of the greater part of the glucose present in the eye-chamber liquid was low and that outside the body a specific rotation of 52·5° could be reached, we then should need at least a 10 cm. tube to be able to investigate the existence of a mutarotation accurately enough, although we should be unable to determine this quantitatively. For technical reasons, tubes longer than 10 cm. cannot be used for micropolarisation. Therefore we lack the ability of reading off a greater rotation and herewith the chance of determining a possible mutarotation with greater nicety.

The tubes were filled with the aqueous humour by means of a glass capillary, as soon as possible after the puncture of the cornea had been made. The first readings were usually made within one or two minutes after the punctures. All readings in these experiments were at room temperature. My eyes, as well as those of the persons who were kind enough to control my readings by making these readings by turns with me, were trained in looking through the polarimeter with the capillary tubes in it. The reading error with these tubes was, when we were well adapted, 0·02–0·03°.

I have done a large number of these experiments with the aqueous humour of normal rabbits. At first we only made as many readings as we could during the first half-hour after the puncture and then took a few readings after a drop of alkali had been added. In later experiments we continued for some hours to make readings at intervals of a few minutes. Because of the great number of such experiments we came to the conclusion, however, that the sugar content of the normal aqueous humour is so small that a possible mutarotation, even qualitatively, could not be determined by this method. We believe, it is true, that we have found with tolerable certainty a few times (in five experiments) that the optical rotation found during the first 5–15 minutes greatly increased, sometimes even to more than 100 % of the original value. That increase differed, however, too little from the reading error which we had fixed. This is possible, as the rotation—not of course the same in all experiments—amounted to an average of only 0·08°. We have never found a pronounced negative original rotation, though we found a few times a value of 0·00–0·02°, which apparently increased to 0·04–0·06°. Comparison of the reduction with the optical rotation has, within the bounds of the reading error, always shown a fairly good agreement. The addition of a trace of alkali to the aqueous humour brought about no distinct alteration in the optical rotation.

It occurs to me, that the doubtful result of the experiments mentioned

above need not be a reason why the use of aqueous humour for an investigation on the nature of glucose in the blood and especially of the stereochemical modifications should be given up. If it were possible to determine the optical rotation of the aqueous humour with greater exactness than is now possible, *e.g.* by using a polarimeter with an objective reading, then the doubt which, I must conclude, now exists concerning the mutarotation of the normal aqueous humour would surely be done away with. Perhaps the optical rotation of the aqueous humour might then also be determined *in vivo*.

The above mentioned investigation of normal aqueous humour was accompanied by a similar investigation of *diabetic* aqueous humour. For this we chose the *aqueous humour after adrenaline injection*. It is shown from the experiments carried out by de Haan and myself [1921, 1, 2] that the hyperglycaemia after subconjunctival adrenaline injection, shows itself only slowly in the aqueous humour, though the increase of the sugar content therein is also very great. In consequence of the results obtained in these experiments, I have now investigated in a number of cases the aqueous humour, two hours after a subconjunctival injection of about 0·75 cc. adrenaline, 1/1000. We also made a qualitative examination of the behaviour towards an alkaline solution of permanganate.

We expected a less rapid decolorisation in connection with the supposition that the diabetic sugar is chiefly a different (stereochemical) modification of glucose than the normal, and with the fact that the behaviour of the normal aqueous humour towards permanganate, as described above, seemed to indicate the presence of a reactive sugar in this liquid. It appeared, however, that *the same permanganate solution became decolorised much more quickly and more intensively by the aqueous humour of the adrenaline injected rabbit than by the normal aqueous humour*. Twenty minutes after the puncture the difference was still clearly to be seen. This difference is probably not to be explained simply by the raising of the sugar content of the aqueous humour, as might be deduced from the control experiments with glucose solutions at similar alkalinity. May we, then, perhaps assume that an equilibrium α-β-glucose $\rightleftarrows$ reactive sugar (γ-glucose?), existing (as supposed by Winter and Smith) normally *in vivo*, is changed after adrenaline injection in the direction of the reactive sugar? The latter would not be in accordance with the hypothesis of Winter and Smith.

The polarimetric investigation, compared with the results of the reduction analyses, might possibly teach us something about this.

Before mentioning the results of such investigations, I wish to draw attention to the fact, that in the literature a test has already been described for distinguishing normal from diabetic blood, which also depends on the reducing properties of the blood (aqueous humour). This test is Williamson's test [Cammidge, 1913]. According to this, methylene blue in alkaline solution is decolorised by diabetic blood (and also by diabetic urine), but not by normal blood. This test, although not wholly in agreement with the difference found in behaviour of normal aqueous humour and this liquid after adrenaline injection, still points in the same direction as regards a possible quantitative proportion between α-β-glucose and the reactive sugar in the normal and diabetic blood.

The result of the polarimetric investigation, compared with the results of the reduction analyses of the eye-chamber liquid after adrenaline injection, have, however, in a large number of experiments, supplied no arguments at all in favour of such an hypothesis, and thus far are more consistent with what Winter and Smith find probable for diabetic blood. The optical rotation now remained, with only a single exception, within the limits of the reading error and this remained so from the moment of the first reading during some hours, after which observations were discontinued. Addition of a trace of alkali did not alter the optical rotation.

The increase of the sugar content, determined by the reduction method, was important in all experiments and in connection herewith also the optical rotation which corresponded closely with the reduction value. The average rotation was $+0{\cdot}22°$. A possible mutarotation of the same nature as formerly supposed might thus have been determined in every case with greater accuracy than in the normal aqueous humour.

The question arises whether any importance as regards a difference in the glucose modification, may still be ascribed to the difference found in the behaviour towards the permanganate of normal and of diabetic aqueous humour. Of course it is possible that disturbance of the equilibrium suggested above, really takes place, but that this is of such a nature, that it cannot be determined polarimetrically. However it may be, these observations teach us again, that one cannot be too careful in drawing conclusions concerning the nature of the glucose modification from the behaviour of the fluid under investigation towards permanganate.

After the experiments on the nature of blood-sugar, described above, were completed, the first publications of Winter and Smith on the same subject came to our notice. The results obtained by these investigators induced me to extend the experiments. The authors have compared the reduction and the polarimetric value of a concentrated protein-free glucose solution, obtained by special technique—which involves a chemical deproteinisation—from blood, under normal and diabetic conditions. In normal men and animals they found that, whereas the reduction of the solution during some days remained constant, the optical rotation increased in three to four days from an original low value, to a constant value, which corresponded with the specific rotation of the α-β-glucose and with the reduction of the solution.

On reading these investigations, we were struck at once by the *slow* change found by Winter and Smith: the equilibrium was only reached after some days. The possibility of such a slow alteration had never been obvious in examining the aqueous humour. This could, however, be connected with the small rotation of the normal aqueous humour that was only examined in a 10 cm. tube. We have therefore examined greater quantities of aqueous humour in ordinary polarimetric tubes of 20 cm. length, and we have extended the time of observation over 4–5 days. Thereupon, we examined *aqueous humour* (in tubes of 10 or 20 cm. length), *of which the glucose concentration was increased by concentration* of the liquid *in vacuo*. And, thirdly, we have extended our research to *ultrafiltrates*—either concentrated or not—*of blood serum and of artificial transudates*.

The greater quantities of eye-chamber liquid were obtained either by puncture of the eyes of several rabbits or from cows' eyes, brought fresh on ice from the abattoir.

For the ultrafiltration a large apparatus of Bechhold was used, with collodion filters of 3 % or 6 % strength. The artificial transudates were obtained by injecting 100–200 cc. NaCl 0·9 % or modified Ringer's solution, of which the composition was given above, into the peritoneal cavity and drawing off the unresorbed liquid, which now contains sugar, after some hours[1]. From this liquid, one obtains a large amount of ultrafiltrate much quicker than from blood serum, and with about the same sugar content as the serum-ultrafiltrate possesses.

The advantage of using a concentrated ultrafiltrate above that of the concentrated aqueous humour lies herein, that the first-named can be kept absolutely free from protein, whilst by the latter with the increase of the sugar-content the trace of protein may also increase to a disturbing concentration. The advantage of the deproteinisation by ultrafiltration over the chemical deproteinisation as followed by Winter and Smith for the subject in question cannot be denied.

Of the various liquids here named we now compared the reduction and optical rotation for some days, and in a large number of experiments. During the time between the several observations the liquids were kept at low temperature.

I have been unable to obtain with the liquids I have used similar results to those which Winter and Smith described for the concentrated protein-free filtrates prepared from normal blood. Time and again I have found that the reduction of the examined liquids remained the same for 4–5 days and that there was also no alteration in the rotation. There was always a small difference between reduction and optical rotation in favour of the former.

We have also had the opportunity of testing an hypothesis suggested by Winter and Smith in connection with their own experiments and with those of other investigators. They have, namely, expressed the possibility that only the glucose form which in normal men and animals forms the greater part of the blood-sugar (the reactive sugar with a small positive or negative rotation) may be used in metabolism. Further, that in the production of this reactive form, insulin may play an important rôle. It seemed to us that the experiments made by Hepburn and Latchford in Prof. MacLeod's laboratory [1922] formed suitable material from which to get support for that hypothesis, and indirectly also for the nature of blood-sugar. These investigators have found, namely, that in perfusing the mammalian heart by Locke's method [Locke and Rosenheim, 1907], the addition of insulin to the perfusion-fluid caused a quantity of glucose to be withdrawn from the perfusion-liquid, which was four to six times as large as the normal quantity. In how far was this due to an increased formation of the so-called reactive form of glucose? We felt justified in putting this question after the well-known experiments of Clark [1916] and others, and of Winter and Smith themselves, although it was not proved that there is actually an increased sugar-consumption under influence of insulin.

Like Winter and Smith, I had found in *in vitro* experiments that insulin alone has no influence on the optical rotation of *d*-glucose in equilibrium. It was possible, however, that the effect found by Hepburn and Latchford was

[1] Compare Feringa [1922].

due to a co-operation of insulin and heart-enzyme *in vivo*, which resulted in an increased transformation of the glucose of the perfusion liquid into the reactive form. If such a process did not take place intracellularly, it might possibly be determined by investigating the perfusion liquid issuing from the heart.

In the perfusion apparatus, at a short distance under the heart, we have therefore fastened a T-piece, through which at any moment a quantity of liquid coming straight from the heart, could be obtained. The reduction and the optical rotation of this liquid were determined at 37°, with or without filtration through a Gooch filter. (When the heart-perfusion lasts somewhat longer, the perfusion liquid always becomes opalescent, as Locke has stated already.)

In the first two experiments, in which no insulin was added, it appeared that the liquid coming from the heart and investigated within one or two minutes afterwards, did not show mutarotation. Then we have in five other experiments, after the perfusion had lasted about one hour, added to the perfusion liquid bit by bit a small quantity of insulin, dissolved in a little perfusion liquid. In three experiments we have found distinctly an increase of the amount of glucose withdrawn from the perfusion liquid, as compared with the normal condition. In no case, however, did the issuing liquid show any mutarotation, either spontaneously or after addition of alkali.

How favourable were the conditions under which these experiments were carried out, may be seen from the following. We thrice observed, that after the heart had contracted regularly for about eight hours and then had stopped beating after the O_2-supply and the warming of the perfusing liquid had been stopped, and was left for about 20 hours at room-temperature, yet the heart began again to beat rather strongly, when the O_2-supply and the warming of the perfusion-liquid were again set going.

Discussion.

In considering the experiments on the nature of blood-sugar described above and judging from these and comparing them with those of other investigators, I must conclude that the question of the stereochemical forms of glucose present in blood under normal and pathological conditions is not yet settled at all. I obtained in my experiments only very small indications about the existence of definite stereoisomeric forms of glucose in normal and pathological blood. Notwithstanding this, the principle of the question, namely, that in blood different stereoisomeric forms of glucose occur with a different biochemical significance, in our opinion need not be given up. The results obtained in those biochemical experiments in which more or less pure solutions of the stereoisomeric sugars were used, such as those of Hamburger [1919, 1, 2; 1921, 1, 2; 1922, 1, 2] and of Hamburger and Brinkman [1918, 1], and the investigations recently published by Willstätter [Willstätter and Sobotka, 1922] give, in our opinion, much support to this conception. That in the case of blood it is so extremely difficult to give direct proofs, may be partly due to technical difficulties, which might be overcome in future, and of which I could mention some.

Then we must remember that the chemical side of the problem is still very complicated. I tried from the beginning to get information about the sugar

of blood principally by comparing the reducing and polarising power of the liquids used, just as had been done by Winter and Smith. Our idea was that the polarimetric evidence alone would be sufficient. This has since been doubted, especially as far as the detection of the so-called γ-sugars is concerned, from the side of the chemists, whereas they also think it highly probable that γ-sugars play an important rôle in carbohydrate metabolism. If their conception is right, then of course we cannot expect to get more information about the presence of γ-sugars in blood as long as we do not know more about the properties and the detection of these sugars in the organism.

The difficulties also concern the question of the mechanism of the action of insulin, if it becomes more probable that this really is connected with a conversion of the stereochemical forms of glucose into each other. Until now, however, this question also seems not to be settled [MacLeod, 1923].

I hope that if the conditions are more favourable in every respect for further research, the methods and results described here may appear to be of some value in obtaining more information about the nature of the normal and diabetic blood-sugar.

Summary and Conclusions.

1. A repetition of Hewitt and Pryde's experiments on the transformation of *d*-glucose in the intestine, with narcotised rabbits and with a dog in which a Vella-fistula was made, has not given any evidence for stereochemical changes of the nature described by these authors.

2. To study the nature of blood-sugar, *aqueous humour* and *serum-ultrafiltrate* have been used. Investigated during the first half-hour after it had been obtained from the eye, the *normal* aqueous humour showed a rapid decolorisation of permanganate directly after the eye-puncture, which decreased in 20–25 minutes in a very important degree. With the technique used, a possible mutarotation of the normal unconcentrated eye-chamber liquid, even qualitatively, could not be determined with certainty.

Reduction and optical rotation of the normal eye-chamber liquid showed a fairly good agreement.

3. After (subconjunctival) adrenaline injection the rapidity of decolorisation by the aqueous humour is increased. With great certainty it may be said that after adrenaline injection the aqueous humour does not show mutarotation of any importance and that the optical value under these conditions closely corresponds to the reduction value.

4. Reduction and optical value of serum-ultrafiltrate, of concentrated serum-ultrafiltrate, of aqueous humour and of concentrated aqueous humour, compared during four to five successive days, may remain the same from the beginning of the experiment, and closely correspond with each other.

5. During the increased removal of glucose from the perfusion-liquid of the heart, observed when insulin is added, no sign of mutarotation can be observed in the issuing liquid, which could point to the formation of a "reactive" sugar.

In conclusion it gives me great pleasure to thank Prof. Hamburger for his constant stimulating help, criticism and advice in the course of these investigations.

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CVII. THE ALLEGED SPECIFIC COLOUR REACTION FOR THE ANTISCORBUTIC FACTOR.

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The disadvantages which attend the biological methods employed in the detection of the vitamins have always kept in the foreground the necessity of devising a chemical means for their estimation. As long as the accessory factors remain chemically unidentified principles it is only possible to fall back on indirect methods, such as colorimetric methods, of estimation.

Funk and Macallum [1913] have demonstrated a certain parallelism between the colour reaction with the Folin and Macallum reagent for the determination of uric acid and phenol and the antineuritic activity of some products. Similarly, Drummond and Watson [1922] have drawn attention to the striking association of the fat-soluble A factor with a substance in oils giving the well-known purple coloration with sulphuric acid. Neither of the above reactions has, however, been found reliable enough to replace the biological methods of estimation of these vitamins.

Recently Bezssonoff [1922] devised a reagent—a modification of Folin's reagent for phenol—which he claimed gave a specific colour reaction for the antiscorbutic factor, because he found that a number of substances known to be antiscorbutically active produced a positive reaction with this reagent.

By employing this reaction on a great number of substances which have been simultaneously tested out on guinea-pigs we have come to the conclusion that, although there is a certain association between this colour reaction and the antiscorbutic activity of some substances, the relationship does not hold true in all cases. We have been able to find, on the one hand, some antiscorbutically active substances which fail to give the colour reaction, and on the other hand, inactive substances which produce the coloration in question.

The colour test was applied by us mostly to various chemical preparations which were tested out biologically in connection with an investigation in which one of us (S. S. Z.) is at present engaged. We soon became aware that although with the majority of the active substances a coloration of varying intensity could be obtained, there was no quantitative relationship between the two tests, and finally, as already mentioned, that it was possible to obtain a biologically active substance giving no coloration with the reagent.

Decitrated lemon juice was prepared in the usual way, namely, by precipitating the acids and other dissolved substances of the juice with excess of calcium carbonate and absolute alcohol, filtering, removing the alcohol in a vacuum at 40–50° in an atmosphere of carbon dioxide and bringing the solution up to the original volume. A daily dose of 1·5 cc. of such a preparation will keep a guinea-pig on a scorbutic diet alive for at least two months. This solution gave a very intense colour reaction. When it was adsorbed with "norit" (5 g. of "norit" to 100 cc. of solution) in a vacuum for 15 minutes (the utmost care having been taken that the adsorbent was introduced in a vacuum after all the dissolved air of the solution had been previously removed by exhaustion) the solution lost some of its antiscorbutic potency, since a daily dose of 1·5 cc. failed to protect a guinea-pig from scurvy; a daily dose of 3 cc., however, prolonged the onset of scurvy, whilst 5 cc. protected the animals for about 52 days, when the experiment had to be discontinued. Only slight scorbutic signs were observed at the autopsy. In spite of the diminished antiscorbutic potency this preparation was, therefore, definitely active, yet no coloration could be obtained with the modified Folin reagent as prepared by Bezssonoff. The above preparation was freshly made up every day before being administered to the animals. During the dosing periods it was tested colorimetrically on eighteen occasions before the animals were dosed and only on three occasions an extremely faint reaction was obtained. On the remaining days the preparation gave negative tests. The tests were carried out, as was done by Bezssonoff, with 1 cc. of the solution diluted with 1 cc. of distilled water to which five drops of the reagent were added. When no coloration was obtained within 30 minutes, undiluted solutions with varying quantities of the reagent were tried for confirmation purposes.

Further experiments showed the possibility of obtaining a positive colour reaction with antiscorbutically inactive substances, viz. yeast and yeast-extract. Brewer's top-fermentation yeast was washed with water and centrifuged. The washed yeast was then dried for 60 hours at 37°. The dried yeast was digested for 3 hours with about ten times its weight of water and centrifuged. On addition of six drops of the reagent to about 2 cc. of the extract a blue coloration was produced immediately with the formation of a precipitate. This precipitate was centrifuged off after standing with the reagent for 10 minutes and a clear blue supernatant solution was obtained.

Similarly, the yeast preparation "marmite" when treated with a few drops of the reagent is precipitated at first and on a further addition of the reagent an olive-green coloration is produced. When the blue coloration obtained with an active substance is compensated in a comparator with untreated marmite the same olive-green coloration is observed.

The above experiments are sufficient to show that the colorimetric test for the antiscorbutic factor suggested by Bezssonoff is not reliable enough to be of any use in the detection of the vitamin. One need only consider the investigation referred to above to see how wrong conclusions might have been arrived

at by depending on the colorimetric reaction alone. The fact that a great number of antiscorbutic substances also give the test is no proof of the specificity of the reagent for the detection of the vitamin.

It is of interest, however, to record that the substance which gives the blue reaction with the modified Folin reagent employed by Bezssonoff is like the vitamin destroyed by oxidation.

Decitrated lemon juice heated at 100° in an atmosphere of carbon dioxide gives the reaction, whilst a similar juice heated in air does not do so. Also a juice made $N/20$ alkaline and allowed to stand exposed to the air for 2 hours at room temperature no longer gives the coloration. This behaviour is similar to that observed [Zilva, 1922, 1923] in the inactivation of the antiscorbutic factor in decitrated lemon juice under the above conditions, which was shown to be due to oxidation.

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CVIII. THE ABSENCE OF EFFECT OF INSULIN ON THE HEAT PRODUCTION IN ISOLATED FROG'S MUSCLE.

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We have carried out a few experiments on the effects of insulin on the heat production of muscle, and although the results so far are merely of a negative character, it seemed to us to be worth while to put them on record since they help at least to restrict the possible methods by which insulin may act upon the system.

For the first four experiments, the insulin used was of the "crude" type, very kindly supplied to us by Messrs Smith and Winter and prepared according to Dudley's method [1923] at the Biochemical Laboratory, Cambridge. Its strength may be taken as being about 5 mg. to the "unit." For the last two experiments, the insulin used was of the "A–B" Brand supplied by the British Drug Co. Its strength is 0·1 cc. to the "unit."

The muscles employed were the sartorius of *Rana temp.*, these being mounted on a thermopile, and the heat production after stimulation being observed by recording photographically the curves of deflection of the galvanometer in series with the thermopile, as fully explained elsewhere [1920, 1922]. The temperature was 14° to 15°. In the present series of experiments, the better to maintain their condition, the muscles were always kept in a salt solution in the thermopile chamber. In this case the actual heat production cannot be calculated from the observed curves, since there is no possibility of making control observations under the same conditions. It is possible, however, by first taking the curves when the muscle is in ordinary Ringer's solution, and then taking another set of curves after insulin has been added, to make a fairly correct estimate of any *change* in the heat production which may be produced by the insulin.

The strength of the insulin in the solution around the muscle was varied between wide limits, but even the weakest solution used would be considered strong if injected into a small animal to make the same concentration in its body.

The chamber containing the muscle was 40 cc. in volume, of which the muscle occupied 0·25 cc.

In Exp. 1 there was 1 mg. of freshly prepared crude insulin in the chamber. In Exp. 2 there were 4 mg. of crude insulin, which had been put into solution on the previous day. In the interval the preparation had been kept on ice.

[1] Working on behalf of the Medical Research Council.

In Exp. 3 there were 4 mg. of freshly prepared crude insulin. In Exp. 4 there were 16 mg. of cude insulin which had been put into solution on the previous day and kept in the interval on ice. In Exp. 5 there was 1 cc. and in Exp. 6 there was 0·5 cc. of insulin "A–B" Brand. The first readings after adding the insulin were taken about an hour after the insulin had been given, and the readings were continued on muscles continuously subjected to the insulin solution for several hours.

Results.

A. *Initial Heat.* Even with the strongest solution of insulin employed the condition of the muscle was apparently unaffected, the initial heat production being practically the same after the muscle had been for five hours in the solution with insulin as it was in the Ringer's solution before insulin was added. Presumably, therefore, the initial breakdown of glycogen or of lact-acidogen into lactic acid is completely unaffected by the presence of insulin.

B. *Recovery Heat.* On comparison of the long continued galvanometer curve for about ten minutes, to see if there were any change in the recovery heat production, a distinct difference was at first detected, showing apparently after insulin had been given that the recovery heat in its earlier stages was less and its later stages more than before insulin. Control experiments, however, were subsequently made on two or three occasions in which long-continued curves of galvanometer deflection were compared (*a*) for a muscle before, and (*b*) for a muscle after being kept in Ringer's solution for several hours. In every case a difference was found between the late and the early curves exactly as between those before and after insulin. This change is presumably due to small alterations in reaction or in the physico-chemical condition of the muscle produced by keeping it in Ringer's solution. There seems to be little doubt, therefore, that even a very strong solution of insulin surrounding an isolated frog's muscle has no noticeable effect upon the oxidative breakdowns involved in the recovery process.

In order to ascertain whether the insulin used has any effect on the frog, six injection experiments with insulin "A–B" Brand were carried out. In two of them 0·25 cc. and in four 0·5 cc. were injected into the dorsal lymph sac. About 24 hours after the injection the frogs were found very weak, relaxed, passive and pale, and in two cases were found dead within 48 hours. By the injection of 0·5 cc. of 4 % glucose solution, the suffering frogs seemed to recover temporarily. By repeated injections of glucose, two frogs recovered completely, while one lived for five days without showing any sign of convulsions. Only one frog (which had been injected with 0·25 cc.) showed proper convulsions. After six days this frog fell into strong convulsions (just like the strychnine convulsions) which ceased promptly on the administration of glucose. On the next day, the convulsions were again produced by touching the frog and were again stopped by glucose. Two days after the frog was found dead.

SUMMARY.

Experiments have been performed on the sartorius muscle of *Rana temp.* comparing (*a*) the magnitude of the initial anaerobic heat production, and (*b*) the magnitude and course of the oxidative recovery heat production before and after subjection to a high concentration of insulin. No change whatever has been found in either case. The injection of insulin, on the other hand, into the intact frog was shown to produce a well-defined effect.

It is clear, therefore, that the action of insulin does not reside in any effect on the muscle, either on its sudden initial anaerobic breakdowns or its slower oxidative processes of recovery.

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CIX. THE RATE OF REPRODUCTION IN ARTIFICIAL CULTURE OF *COLPIDIUM COLPODA*. PART II.

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In a previous paper [Cutler and Crump, 1923] an account of experiments on mass cultures of *Colpidium colpoda* was given; the present communication deals with similar experiments carried out on cultures containing one or more cells isolated into one cubic centimetre or less of culture solution. The objects of these experiments have been to discover whether the results obtained from cultures containing few organisms in small quantities of fluid are comparable with those in which numerous animals are inoculated into comparatively large amounts of liquid, 10 cc. or more. Also there have appeared a series of papers by Robertson [1921, 1922], describing new and interesting phenomena —allelocatalysis, etc.—in cultures of a ciliate *Enchelys farcinem*. These seemed to be of so fundamental a character that it was felt desirable to test whether they obtained with other species of ciliates.

Methods.

The medium used has been the synthetic one in which the experiments on mass cultures were conducted. The smaller cultures (*i.e.* less than 0·09 cc. in volume) were put up as follows in unruled counting chambers 0·1 mm. deep: the individual, or individuals, are transferred, by a capillary tube from the parent culture, with about 0·01 mm.^{3} of liquid, to the chamber. It has not, however, been possible to keep this quantity rigidly constant, owing to the small volume of the fluid used; the chamber is then covered with a thick cover slip, on which there is already a drop of new medium, freshly inoculated with *Sarcina* to provide an adequate food supply, and the two drops are allowed to mix. The final size of the drop may vary from 0·37 to 9·8 mm.^{3}, and the degree of dilution varies between 1 in 37 and 1 in 980; these dilution figures, however, are only an approximation owing to the difficulty of measuring the original minute drops containing the organisms with accuracy, and, for the same reason, the size of the drop is not of necessity an index of the degree of dilution. Before the cover slip is put on the chamber a ring of small drops of sterile medium are placed in the groove around the raised central part to discourage

evaporation from the culture itself. The drop size in any culture is measured by projecting an image of the chamber on to squared paper, where the outline of the drop is drawn; as the magnification and the depth of the liquid (0·1 mm.) are both known, by counting the squares enclosed by this outline, the volume can be calculated. Each culture has been measured in this way every day so that changes in volume due to evaporation or condensation are detected. The numbers of animals present are counted by projecting the image of the chamber on to a screen, using a 48 mm. lens, and adjusting the eye-piece and distance of the screen so that the final magnification is from 50 to 60 diameters. With practice numbers of animals up to 100 can be counted with a sufficient degree of accuracy.

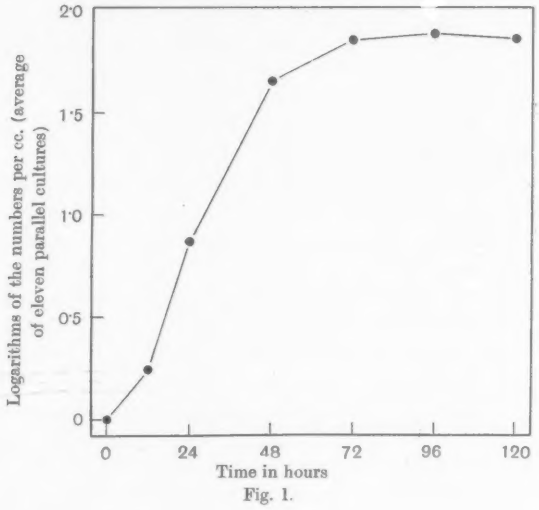

Fig. 1.

Results.

The curves obtained by plotting the numbers of animals in these single cell cultures against the time are similar to those derived from mass cultures, at least until the maximum numbers are reached; after this point, although some of the cultures show the same oscillations that occur in mass cultures, a certain number die out completely in 24 hours. Fig. 1 shows the curve obtained by averaging the numbers of animals every day in eleven parallel cultures (I series, 1 day parent), each starting from a single cell, and plotting the logarithms of these numbers against the age in hours (cf. Fig. 2 in previous paper). It will be seen that the sequence of events in these single animal cultures is in the main similar to that in mass cultures.

Allelocatalysis.

To test the allelocatalytic effect described by Robertson [1921, 1922] a number of experiments were made in which one, two, three or four animals were inoculated into drops of various sizes. The cultures were examined, usually after 12 hours' incubation, and then daily until discontinued. In Tables I and II are given the rates of reproduction arranged in order according to the size of the drop or to the equivalent number of animals inoculated per cc. of liquid. It will be seen that we have been able to find no evidence of the effect obtained by Robertson; for, according to his results, the reproductive rate should increase as the number of animals inoculated increases (Table I); and, the greater the initial concentration per cc., the greater the rate of reproduction should be (Table II). Nor does the size of the drop, within the limits of the experiment, appear to have any appreciable effect, though there is an indication that the animals in the larger drops (giving a low initial concentration) are capable of reproducing a little more rapidly than in the converse case. This would also appear to be in direct opposition to Robertson's theory of the necessity for the animal to develop a definite concentration of "X substance" before division can take place. It should be noted, however, that Robertson [1921] worked with a much larger drop than we have found possible (0·08 cc.) and when only one animal was inoculated the concentration would be only 12·5 per cc. This would materially affect the experiment, but, as *Enchelys farcinem*, the organism with which he worked, measures 50–70μ long by 35–50μ broad, while our variety of *Colpidium colpoda* is only about 45–55μ × 10–20μ[1], the amount of protoplasm in a drop of the size that Robertson used and the amount in our large drops would not be markedly different. If, then, the quantity of "X substance" produced is at all proportional to the amount of protoplasm producing it, Robertson's results and ours are at variance. It is realised, however, that such an argument may be erroneous, but we would emphasize the fact that the "allelocatalytic effect" does not obtain with *Colpidium colpoda* isolated in drops of the varying sizes we have used. In his last paper, Robertson [1922] states that with *E. farcinem* "single individuals isolated into volumes exceeding 1 cc. very rarely survive and failures to reproduce on the part of individuals isolated into cultures exceeding 0·1 cc. in volume are not at all infrequent." This, as the author points out, is a common experience with micro-biologists.

Assuming that it is due to the inability of the organism, *E. farcinem*, to produce a sufficient concentration of the X substance to induce reproduction, a smaller animal such as *C. colpoda* ought to be incapable of division when isolated into 1 cc. of culture fluid. Experiments were therefore made to test this hypothesis. Here single animals were isolated into 0·25 cc. (4 per cc.), into 0·33 cc. (3 per cc.), into 0·5 cc. (2 per cc.) and into 1 cc. (1 per cc.); two animals were also isolated into 0·25 cc. (8 per cc.) and into 0·5 cc. (4 per cc.) of

[1] The sizes of the animals given in the footnote on p. 179 of our previous paper [Cutler and Crump, 1923] should be 30μ × 10·5μ and 38·9μ × 21·6μ respectively.

Table I. *Table showing the reproductive rates of* 1, 2, 3 *and* 4 *animals during the first* 24 *hours after inoculation in drops of varying sizes.*

Size of drop in cubic mm.	1 animal inoculated	Av.	2 animals inoculated	Av.	3 animals inoculated	Av.	4 animals inoculated	Av.
0–0·5	—	—	2·4, 2·7	2·55	—	—	—	—
0·5–1	2·0, 0·0, 1·0, 1·0, 2·0 2·0, 3·5	1·64	2·3, 0·7, 1·8, 2·5, 1·0 1·8, 2·1, 2·5, 3·2	1·99	1·6, 1·7, 3·2, 2·1, 2·4	2·20	1·7, 1·7, 3·3, 1·3	2·00
1–1·5	2·0, 2·0, 3·0, 2·0, 1·0 1·0, 2·0, 0·0, 3·9, 3·8 3·3, 2·0, 2·0	2·15	3·6, 2·3, 3·6, 3·9, 2·3 3·7, 2·5, 2·5	3·05	1·9, 2·0, 2·9, 2·7, 0·7 2·5, 3·8, 3·6	2·51	1·8, 3·3, 3·1	2·71
1·5–2	3·0, 4·0, 1·0, 2·0, 3·1 1·5, 1·0	2·23	1·0, 3·4, 2·5, 3·6	2·62	3·0, 2·8, 1·0, 2·9, 3·7 2·5, 3·4, 3·6, 2·1, 1·2 1·6, 2·6	2·53	2·6, 3·6, 2·0, 2·5	2·67
2–2·5	2·0, 3·9, 1·0, 1·0	1·97	3·8, 2·7, 2·0, 1·5, 1·3	2·26	2·2, 2·3, 3·4, 1·6, 1·9 1·6	2·16	3·3, 2·0, 2·2	2·83
2·5–3	3·0, 3·0	3·00	1·0, 3·4, 2·3, 1·3	2·00	1·4	1·40	1·5	1·50
3–3·5	3·0	3·00	—	—	2·9, 2·7, 4·1, 2·0	2·92	2·7, 3·5, 3·5, 1·3	2·75
3·5–4	1·5, 4·0, 4·4, 4·6, 3·8 2·0	3·38	—	—	2·3, 1·7	2·00	—	—
4–4·5	2·0, 3·0, 2·0, 3·0	2·50	—	—	4·1	4·10	1·7, 2·4	2·05
4·5–5	3·9, 1·0	2·45	3·0	3·00	—	—	2·8, 2·1	2·45
5–5·5	2·0, 2·8	2·40	4·5	4·50	1·0	1·00	—	—
5·5–6	2·0, 3·3, 3·1	2·80	—	—	1·4	1·40	—	—
6–6·5	—	—	2·1, 3·8	2·95	—	—	—	—
6·5–7	3·7, 1·5	2·60	—	—	—	—	—	—
7–7·5	—	—	4·3, 1·0	2·65	—	—	—	—
7·5–8	—	—	—	—	—	—	3·3	3·30
8–8·5	3·4	3·40	3·0	3·00	—	—	—	—
8·5–9	—	—	—	—	—	—	3·3	3·30
Average for drops of all sizes		2·4		2·5		2·4		2·5

Table II. *Table showing the reproductive rates during the first* 24 *hours in cultures whose initial concentrations vary from* 100 *to* 8000 *per cc.*

Number of animals per cc. inoculated	Reproductive rate for the first 24 hours	Av. rate of reproduction for the first 24 hours
100– 200	2·0, 3·9, 3·3, 2·0, 2·5, 3·1, 3·4, 2·5, 3·7	2·96
200– 300	2·5, 1·0, 1·0, 2·0, 3·9, 3·0, 1·0, 3·0, 3·0, 3·8, 2·0, 4·0, 4·0, 4·2, 4·5, 1·0, 2·0	2·70
300– 400	3·9, 3·0, 3·0, 2·1, 3·8, 4·5	3·39
400– 500	2·0, 2·0, 1·0, 3·3, 3·0, 1·0	2·05
500– 600	3·0, 0·0, 4·0, 2·5, 1·0, 3·3, 2·0, 1·0, 1·0, 1·4	1·92
600– 700	2·0, 1·8, 3·9, 3·1	2·70
700– 800	2·0, 3·0, 1·0, 1·5, 1·0, 2·3, 1·0, 2·0, 3·4, 4·1, 3·3, 2·3	2·23
800– 900	1·0, 2·0, 3·7, 2·9, 2·8, 2·7, 2·0, 2·4, 1·7, 2·0, 2·0, 2·5, 1·7	2·23
900–1000	1·0, 0·0, 1·0, 2·0, 1·0, 1·0, 3·8, 2·7, 1·7, 4·1, 2·0	1·84
1000–1100	3·5	3·50
1100–1200	2·5, 2·7, 1·4	2·20
1200–1300	3·3, 2·2, 3·6, 3·5, 3·6, 3·6, 3·4, 1·8, 1·5, 1·3	2·78
1300–1400	1·0, 2·0, 1·7, 1·6	1·57
1400–1500	2·0, 2·3	2·15
1500–1600	2·0, 1·0, 3·7, 2·3, 3·6, 2·5	2·51
1600–1700	1·0, 3·5, 2·5, 3·7, 3·3, 2·6	2·76
1700–1800	2·9, 2·1, 1·6	1·40
1800–1900	2·9, 2·1, 1·6, 1·0, 3·0	2·12
1900–2000	0·0, 2·5, 3·6, 2·0, 3·3, 3·8, 3·1, 2·1, 1·2, 2·5	2·41
.....		
2000–3000	2·0, 2·6, 3·5, 1·0, 0·7, 2·5, 1·8, 1·9, 3·3, 3·1, 0·7, 2·1	2·10
3000–4000	2·4, 0·5, 2·3, 1·8, 1·0, 2·5, 3·1, 1·8, 2·1	1·94
4000–5000	3·0, 1·2, 2·7, 1·3	2·05
5000–6000	0·0, 2·4, 1·6, 1·6, 1·7	1·46
6000–7000	1·0	1·00
7000–8000	3·2	3·20

liquid. The medium used in these experiments was one in which 0·024 % of ammonium phosphate and 0·016 % of glycerol were substituted for the sodium phosphate, ammonium lactate and glucose of the medium used in the other experiments. Where only one animal is present any growth must be due to that animal, but where two are introduced there is always the possibility that one may have died. Attempts were made to find the animals immediately after inoculation, and also to count them by direct examination of a projected image of the culture tube; this can only be very unsatisfactory even in 0·25 cc. cultures, though by using small tubes (2 × 0·3 inches), and immersing them in water to lessen refraction, a satisfactory image can be obtained and a rough approximation to the number of animals made. When the numbers rise to 400 per cc. or more, counts are made in a counting chamber.

The following table shows the figures obtained.

Table III.

Initial no. per cc.	No. of parallels	Average reproductive rate
1	5	14·94
2	5	13·80
3	5	13·60
4	7	12·72
8	2	11·05

These results show that the organism is capable of vigorous reproduction even when isolated into relatively large volumes of liquid[1].

Acceleration due to X substance.

The experiments detailed above demonstrate that the action of the growth promoting substance X is not so simple as might be thought by a perusal of Robertson's communications; but the work of this author and of others leaves little doubt but that some kind of accelerative agent is produced during the growth of organisms in culture fluid.

Since the publication of Wildiers' paper [1901] on the growth of yeast cells, where it is shown that the yeast does not develop its optimum reproductive capacity in the absence of an unknown substance, termed by Wildiers "bios," many investigations have been made to determine the nature of the accelerative agent. This work need not be detailed here as already many summaries have been made. It may be recalled, however, that Cantani [1901] reports that the growth of *Bacillus influenzae* is enriched by the presence of *C. diphtheriae*, *M. gonorrhoea* and certain staphylococci; a result also obtained by the use of dead bacteria as the stimulating agent. Such observations have been confirmed and extended by Neisser [1903] and Thjötta [1921, 1, 2]. Very few observations have been made on organisms other than bacteria, though Haberlandt [1913, 1920] adduces evidence that the plant cell produces a substance that is capable of inducing rapid cell division.

In view of the importance of these conclusions we have performed similar

[1] That single bacteria are also able to grow when inoculated into relatively large volumes of medium has been shown by Barber [1908], Churchman and Kahn [1921], and others.

experiments on *Colpidium colpoda*. The results, given in Table IV, show that crushed *Sarcina* or crushed *Colpidia* have the power of accelerating cell division; though the action does not seem to occur during the first 24 hours of incubation. This is contrary to the findings of Robertson, and is difficult to understand on the analogy of enzyme action.

Also the possibility has not been excluded that the increased reproduction is due to an increased food supply afforded by the crushed fragments of protozoa or bacteria. Such a possibility, however, obtains also in Robertson's experiments.

It is our intention considerably to extend these experiments, but in view of the interest of the problem it seems worth while to record the few observations already made.

Table IV. *Showing the accelerative effect due to the presence of crushed* Sarcina *and crushed* Colpidium.

Reproductive rate at the end of 24 hours' growth.

Initial concentration	Control	Av.	Crushed *Colpidium*	Av.	Crushed *Sarcina*	Av.
100– 500	1·0	1·00	—	—	1·0, 1·0	1·00
500–1000	1·0 1·0, 0·0, 1·5, 1·0, 0·0	0·75	1·0, 2·0, 1·5, 1·0, 0·0, 1·7	1·20	0·1, 0·0	0·05
1000–1500	1·0	1·00	1·4, 1·0, 1·0	1·13	—	—
1500–2000	0·0	0·00	0·0	0·00	—	—
2000–2500	1·0	1·00	0·1	0·10	—	—
2500–3000	0·5	0·50	1·4	1·40	—	—
.....						
5000–5500	0·0	0·00	—	—	—	—
6000–6500	1·0	1·00	—	—	—	—
8000–8500	—	—	1·0	1·00	—	—
Average:	0·69		1·00		0·52	

Reproductive rate after 48 hours' growth.

100– 500	2·0	2·00	—	—	4·8, 3·9	4·35
500–1000	1·0, 2·0, 2·0, 2·9, 2·0, 1·0	1·81	3·8, 4·5, 4·2, 1·8, 2·0, 3·3	3·26	3·7, 4·6	4·15
1000–1500	1·0	1·00	4·3, 1·0, 2·9	2·73	—	—
1500–2000	0·0	0·00	0·0	0·00	—	—
2000–2500	1·5	1·50	2·8	2·80	—	—
2500–3000	0·7	0·70	2·5	2·50	—	—
.....						
5000–5500	0·0	0·00	—	—	—	—
6000–6500	1·4	1·40	—	—	—	—
8000–8500	—	—	3·1	3·10	—	—
Average:	1·34		2·78		4·25	

In connection with the accelerative effect of extracts of *Colpidia* and *Sarcina* it is interesting to note the action produced by the inoculation of fluid from a culture which had grown well into one which had shown a low rate of reproduction. The organism on which this test was made was a flagellate, *Oicomonas termo*. On May 15th, 1922, there was inoculated into 20 cc. of ammonium phosphate + saccharose medium sufficient *Oicomonas* to give a concentration of 70,238 per cc. The culture (*Z* 2) during the first 24 hours decreased in numbers to 51,500 per cc., but at the end of 72 hours had increased to 2,575,000 per cc.; thus showing a reproductive rate of 5·6. Concurrently with this another culture, *X* 1, was started in the same amount of culture fluid but with a concentration of only 17,559 per cc. During the first few hours of incubation the numbers fell to 7000 per cc., and at the end of 72 hours had

reached 13,000, showing a reproductive rate of only 0·89. At this juncture, therefore, a portion of *Z* 2 was centrifuged to eliminate the organisms, and 1 cc. of the supernatant fluid was added to *X* 1*a*, a culture formed by dividing *X* 1 into two equal 10 cc. portions.

Hours:	0	4	24	48	96	120	144
X 1	13,000	9,500	2,000	2,000	under 1000	under 200	under 200
X 1*a*	13,000	17,500	31,000	20,000 + cysts	21,000	15,000	2,000

From the above table it is seen that, while the control culture *X* 1 steadily decreased in numbers, the treated *X* 1*a* culture, after 4 hours, exhibited the effect of the treatment, and after 24 hours had had a reproductive rate of 1·25.

The experiment is also of interest in demonstrating that the decrease in numbers in an old culture is not primarily conditioned by the culture fluid becoming toxic: for the numbers of *Z* 2, whose culture fluid was added to form *X* 1*a*, steadily fell after the beginning of the experiment. This conclusion has been independently arrived at by Robertson from other evidence, and was also put forward by us in our previous paper.

Rate to first maximum.

In our previous paper it was shown that, when the logarithms of the numbers inoculated into a constant volume of medium were plotted against the maximum numbers attained, a reproductive rate varying from five to six divisions was found for 91 % of the cases. This held for inocula of 100 to 700 per cc., for parent cultures from 24 to 48 hours old, and the time required for the divisions varied from 48 to 168 hours.

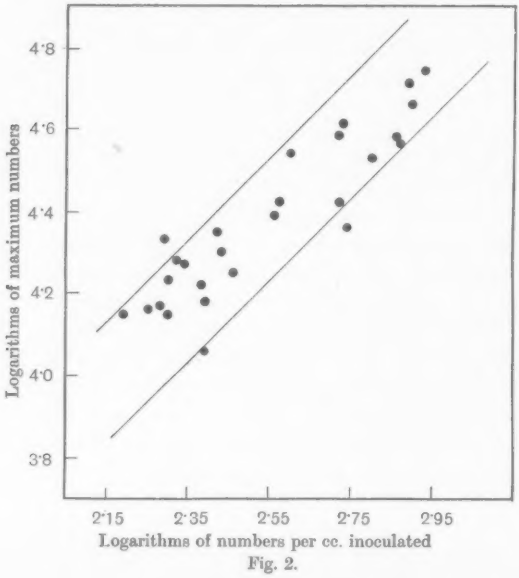

Fig. 2.

In the single cell experiments described above the majority of the cultures were not kept sufficiently long to attain their maximum numbers, but in 29 cases this was done. When the procedure adopted for the mass cultures is used it is found that again the reproductive rates fall within narrow limits, but instead of being from 5 to 6 divisions they are from 5·5 to 6·5 divisions (Fig. 2). This obtains, however, only when the initial concentration is equivalent to numbers lying between 100 and 900 per cc. Within these limits the age of the parent culture may be 24 or 48 hours, and the time taken to reach the maximum has varied from 72 to 168 hours. With concentrations above 900 per cc. the reproductive rates fall rapidly, as is shown in the following table:

Culture	Initial concentration per cc.	Rate	Final concentration per cc.
F 4	906	3·00	8,384
H 10	912	5·46	37,620
G 8	920	5·42	40,334
H 2	981	4·80	39,236
G 2	997	4·08	17,459
G 10	1,560	4·00	23,850
H 19	1,686	4·50	40,894
I 4	1,704	3·93	25,856
H 18	1,720	3·70	23,582
B 17	1,857	3·62	30,784
B 10	2,380	3·70	34,700
D 1	2,475	1·50	8,534
G 12	2,838	1·73	12,507
D 2	3,220	0·80	5,220
E 2	5,292	0·00	5,292
E 6	6,116	1·10	11,830

It was thought possible that the high concentration giving a low reproductive rate might be explained as follows: suppose each organism was capable of dividing 5 times, and, that, at a certain concentration x, autointoxication occurred; then, it might be, that when the initial concentration was high, the critical concentration x was obtained before 5 divisions had occurred. This, however, will not explain the result, since a culture *G*5, which had initially two animals and a concentration of 848 per cc., divided 5·9 times and attained a final concentration of 54,570 per cc. From the above table it will be seen that the final concentrations, in those cultures showing a depressed rate of reproduction, are below that of *G*5; though in the majority of cases if an extra division had taken place the concentration would have been above or near to that found in *G*5. Also with concentrations considerably below 100 per cc. the rate of reproduction does not fall within the limits 5·5–6·5, but is considerably increased as is shown in Table III.

These results indicate that with inocula of this size the animals tend to reach a constant maximum concentration instead of dividing a definite number of times as they seem to do when the inocula lie between 100 and 900 per cc. This tendency to arrive at a constant population is also seen when part of a mass culture is centrifuged to reduce the number of animals, and the supernatant fluid poured off. The reproductive rate of the animals left in this fluid is higher than it is in those that remain in the original culture.

SUMMARY.

The rate of reproduction of *Colpidium colpoda* has been tested in cultures derived from one or more animals isolated into small volumes of fluid. It is shown that in the main such cultures are comparable with mass cultures.

The allelocatalytic effect, described by Robertson, has been tested for and found not to obtain with *Colpidium* when isolated into fluid whose volume varies from 0·5 to 8·5 mm.^{3}. A few experiments are given in support of the contention that the rate of reproduction can be accelerated by the addition of small quantities of crushed bacteria or protozoa.

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CX. A PRELIMINARY NOTE ON THE DESTRUCTION OF VITAMIN B BY AGE.

By GEORGE MARSHALL FINDLAY.

From the Royal College of Physicians' Laboratory, Edinburgh.

(*Received October 30th, 1923.*)

Although much work has been carried out on the destruction of vitamin B by such agencies as heat and oxidation, comparatively few investigators have examined the question of the loss in the vitamin B content of natural foodstuffs caused by the mere passage of time.

Vedder and Williams [1916] found that unpolished rice stored in a warm moist place for one year retained its power of curing polyneuritis in pigeons. While this paper was in course of preparation there appeared a communication by Jansen [1923] who states that a sample of rice which had been stored as paddy for 100 years was found to have lost, as compared with new rice, little or none of its anti-beriberi vitamin. The question as to whether the anti-beriberi vitamin is identical with the vitamin B, growth-promoting factor is still unsettled. In the present paper a preliminary account is given of investigations on the vitamin B content of seeds stored under constant conditions for a number of years.

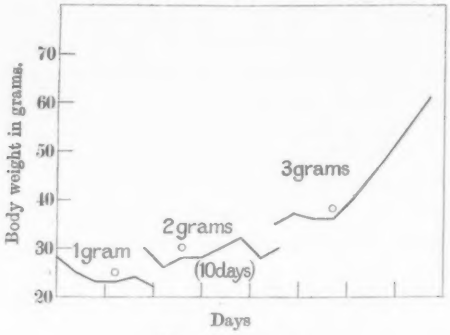
Through the kindness of Professor Watson of the Department of Agriculture of the University of Edinburgh, I was provided with a supply of certain Indian lentils which are known to have arrived in this country in the spring of 1886. They are thus at least 38 years old. During the whole of this period they have been stored in canvas bags in a dry cupboard in the Department of Agriculture. All the seeds had lost the power of germination. The different lentils examined included:

1. *Cicer arientinum* Linn.—"Butt" (yellow variety).
2. *Phaseolus mungo* Linn.—"Sona Moona" (golden yellow in colour).
3. *Cejanus indicus* Spreng.—"Ahar" (yellow-ochre variety).
4. *Phaseolus radiatus* Linn.

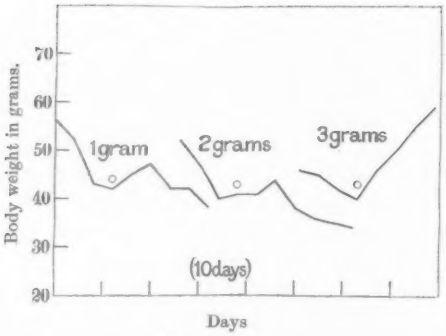
Unfortunately, it was not possible to obtain supplies of fresh lentils for comparison with these seeds of 1886, but luckily, their vitamin B content has recently been investigated by Ghose [1922]. Although no data are given by him as to the age of the seeds used, it is to be presumed that they were not more than two or three years old. Ghose found that the addition of 1 g. of any of the above lentils to a standard diet lacking vitamin B was sufficient to promote adequate growth in rats. No observations, however, were made as to whether 1 g. was the minimal amount necessary to ensure growth.

In order to compare my results with those obtained by Ghose, the technique adopted was closely similar, except that all faeces were removed from the cages twice daily, since by devouring their own faeces it is possible that rats may obtain small supplies of vitamin B. The rats used were slightly lighter than those employed by Ghose—the average weight at the beginning of the experiment being about 40 g. The results obtained by the addition of 1, 2 and 3 g. of lentils to the standard diet lacking vitamin B are shown in the charts, where each curve represents the average weight of four rats. It will be seen that growth occurred after the addition of 3 g., but not after the addition of 1 or 2 g.

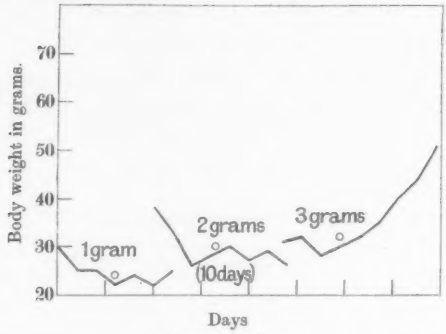
Experiments were also carried out with *Pisum sativum* which had been stored for 38 years. Here, again, the addition of 3 g. to the standard diet gave adequate growth, while no growth was obtained by the addition of 1 or 2 g. of the pea. The power of germination had been entirely lost.



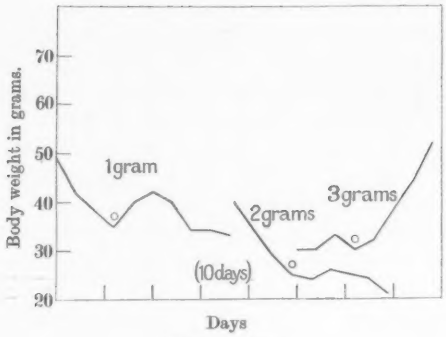
Phaseolus mungo.



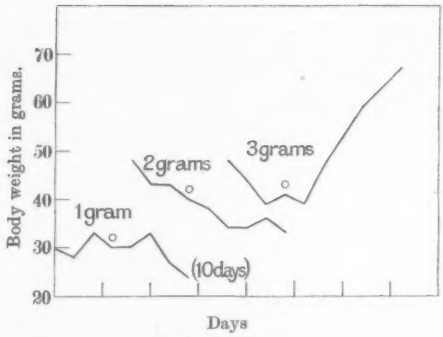
Cicer arientinum.



Phaseolus radiatus.



Pisum sativum.



Cejanus indicus.

Conclusions and Summary.

Samples of Indian lentils and peas kept for 38 years still contain appreciable quantities of vitamin B. In comparison with the observations of Ghose, however, they appear to have lost a small amount of their vitamin B content, even if allowances are made for the slight differences in technique previously described.

Seeds which have lost the power of germination have not necessarily lost all their vitamin B content.

The whole question of the destruction of vitamin B with age is obviously one which requires further and fuller investigation, more especially in regard to the effects of different climatic conditions, *e.g.* heat and moisture, on the rate of destruction.

I desire to express my thanks to Professor Watson and Dr Smith for their kindness in supplying me with seeds, as also to Lieut.-Col. McKendrick for his continued interest in this research.

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CXI. THE ABSORPTION SPECTRA OF SOME INDOLE DERIVATIVES.

By FRED WILBERT WARD.

From the Biochemical Laboratory, Cambridge.

(*Received August 8th, 1923.*)

In the course of an investigation on the metabolism of tryptophan and some of its derivatives, it was thought advisable to study the absorption spectra of several of these compounds.

Apparatus.

The apparatus used for the measurement of these absorption spectra was a quartz ultra-violet spectra photometer made by Adam Hilger, Ltd. It consisted of a quartz ultra-violet spectragraph with an electrode holder, a 1/4 kilowatt transformer, a suitable condenser and a rotating sector photometer. The spectragraph took a plate 10 × 4 inches and also contained a wave length scale mounted inside the spectragraph. By means of this scale the wave length could be photographed on the plates.

For electrodes, nickel rods were found to be the best for this kind of work. The emission spectrum of nickel is nearly as rich in lines as that given by iron and continues as far down as 2200 A.U. whereas the spectrum of iron has relatively few lines below 2500 A.U. Copper gives an emission spectrum which goes as far down as 2100 A.U. but the lines are not nearly as plentiful and it does not give nearly as satisfactory results in the measurement of absorption spectra.

Method.

In the Adam Hilger spectra photometer, simultaneous adjacent pairs of photographs of the spark across the electrodes are taken, one of which is through a cell containing the solution of the material to be measured and the other through a cell containing the solvent. The photograph through the solvent is to be considered the standard with which the other is to be compared. A number of such pairs of photographs are taken and at the finish of each exposure the sector wheel in front of the solvent or standard is closed down, the amount of light allowed to pass through by the sector wheel unto the photographic plate being in a definite ratio to the light that would pass through the solution providing no absorption were taking place. After the plate has been completely exposed, developed, washed and dried, the point of equal blackening is marked on each of the simultaneously taken adjacent pairs of photographs.

The absorption curves are plotted on cross section paper in which one ruling is arithmetic and the other logarithmic. The wave length is plotted on the arithmetic scale and the molecular absorption coefficient on the logarithmic scale. The molecular absorption coefficient is calculated from the following equation.

$$M = \frac{1}{d} \times \frac{\log I_0 - \log I}{C}.$$

The Adam Hilger sector photometer is graduated to read in terms of

$$\log I_0 - \log I.$$

Hence the readings on the sector wheel may be called α and the equation then becomes

$$M = \frac{1}{d} \times \frac{\alpha}{C},$$

where M is the molecular absorption coefficient,
d is the thickness of the absorbing layer in centimetres,
$\log I_0$ is the logarithm of the wave-length of the incident light,
$\log I$ is the logarithm of the wave-length of the transmitted light,
and C is the molecular concentration.

Experimental.

In all ten different compounds were measured. These ten compounds form four groups.

Group I: Indole, β-indolecarboxylic acid, and β-indole-aldehyde.

The indole was obtained from one of the drug houses. It was carefully purified by boiling with animal charcoal and recrystallising from dilute alcohol.

The β-indolecarboxylic acid was prepared by means of the Grignard reaction [Majina and Kotake, 1922]. It was recrystallised by dissolving in acetone and pouring into water [Ellinger, 1906]. This gave a white crystalline solid melting at 213°.

The β-indole-aldehyde was prepared by oxidising tryptophan with ferric chloride [Hopkins and Cole, 1903; Ellinger, 1906]. It was boiled with animal charcoal and recrystallised from dilute alcohol.

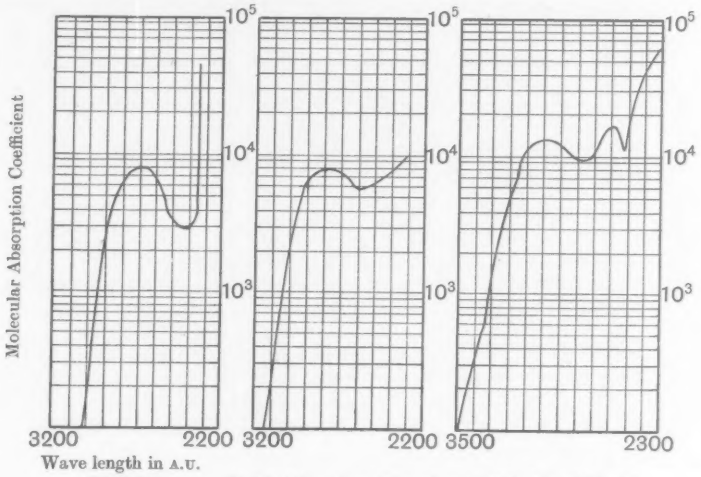
Fig. 1 shows the absorption curves of these compounds.

Group II: β-Indole-ethyl alcohol, β-indolepropionic acid and β-indole-aminopropionic acid or tryptophan.

The β-indole-ethyl alcohol was prepared from tryptophan by the action of yeast fermentation [Felix Ehrlich, 1922]. It was decolorised by boiling with animal charcoal and carefully recrystallised from very dilute alcohol.

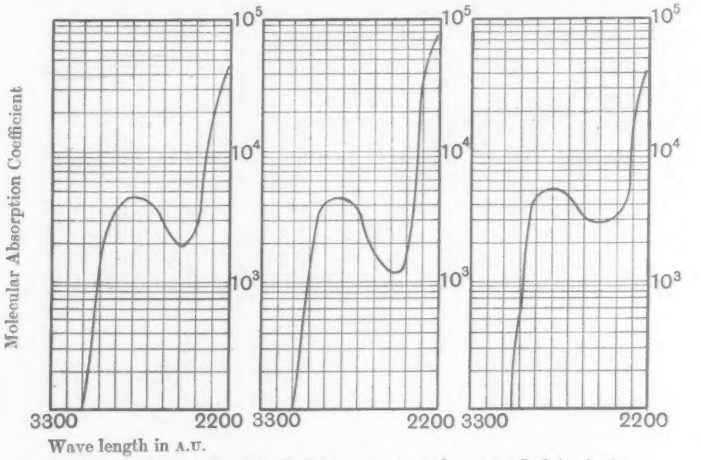
The β-indolepropionic acid was prepared from tryptophan by the action of anaerobic bacteria under anaerobic conditions. The bacteria used were a mixture of *B. coli*, *B. chauvei*, *B. sporogenes* and *B. oedemaciens* [Nencki, 1889; Hopkins and Cole, 1903]. The introduction of the four different strains is a modification of the previous methods and will be published in more detail elsewhere. The indolepropionic acid was carefully decolorised with animal charcoal and recrystallised from dilute alcohol.

The tryptophan was prepared by the original method of Hopkins and Cole [1901]. It was very carefully decolorised with animal charcoal and recrystallised from 65 % alcohol.



(*a*) Indole. (*b*) β-Indolecarboxylic acid. (*c*) β-Indole-aldehyde.

Fig. 1.



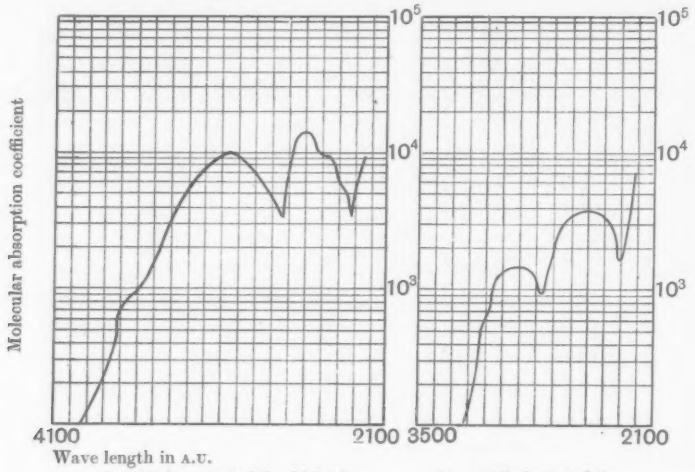
(*a*) β-Indole-ethyl alcohol. (*b*) β-Indolepropionic acid. (*c*) β-Indole-alanine or tryptophan.

Fig. 2.

Fig. 2 gives the absorption curves of these compounds.

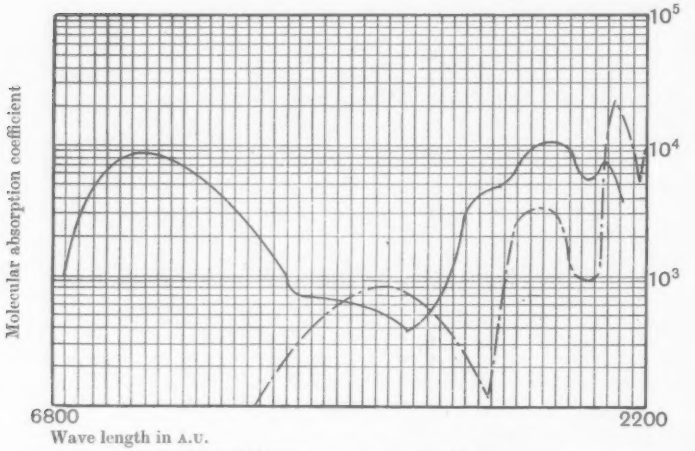
Group III: α-β-Dihydroxyindole and α-hydroxy-β-indole-aldehyde.

The α-β-dihydroxyindole was prepared from isatin by reducing with sodium hydrosulphite [Marschalk, 1912]. It was carefully decolorised with animal charcoal and recrystallised from water.



(*a*) α-Hydroxy-β-indole-aldehyde. (*b*) α-β-Hydroxyindole.

Fig. 3.



Sodium indigosulphonate ———. Isatin — - — - —.

Fig. 4.

The α-hydroxy-β-indole-aldehyde was prepared from thio-indigo Scharlach, R. Kalle and Co. [Friedländer and St Kielbasinski, 1912]. It was boiled with animal charcoal and recrystallised from dilute alcohol. Pale yellow crystals were obtained melting at 208°.

Fig. 3 shows the absorption curves of these compounds.

Group IV: Isatin and sodium indigosulphonate.

The isatin was obtained from a drug house and carefully boiled with animal charcoal. It was then recrystallised from dilute alcohol.

The sodium indigosulphonate or indigo carmine was a sample of Kahlbaum's pure chemicals and was used without further purification.

Fig. 4 shows the absorption spectra curves.

Discussion.

In examining the charts of group I, namely indole, β-indolecarboxylic acid and β-indole-aldehyde, one may observe the effect of substituting carboxyl and aldehyde groups in the β-position of the indole ring. Indole has a band with the tip at 2700 A.U. and with a molecular absorption coefficient of about 9000. The effect of substituting a carboxyl group in the β-position is to shift the band about 100 A.U. towards the red end of the spectrum but the intensity of the absorption is not changed. The effect of substituting an aldehyde group in the β-position is different. The band is also shifted towards the red end of the spectrum but the shift is much greater, being about 400 A.U. or four times that due to the carboxyl group. The intensity of the absorption is also increased from a molecular absorption coefficient of 9000 to 15,000.

V. Henri [1912] has shown that the carboxyl group of the aliphatic acids, such as acetic and propionic acid, absorbs the rays in the region of 2200 to 2400 A.U., the intensity increasing and shifting towards the red with increasing molecular weight of the substance. This same type of absorption may be observed in β-indolecarboxylic acid. Henri has also shown that aliphatic aldehydes such as acetaldehyde and propylaldehyde have a characteristic type of absorption curve. These curves rise to a peak around 2600 A.U., fall off again and then rise again in the extreme ultra-violet around 2200 A.U. This same type of curve is found in the lower end of the absorption spectrum of β-indole-aldehyde. Fig. 1 *c* shows this type of curve.

In examining the charts of group II, which consists of β-indole-ethyl alcohol, β-indolepropionic acid and β-indole-aminopropionic acid, it will be observed that the tips of the bands of all three are at about the same molecular absorption coefficient. It should be noted that these three compounds are substituted compounds of β-ethylindole, the substitution being in the side chain and being firstly a hydroxyl group, secondly a carboxyl group and thirdly an amino and a carboxyl group.

The point to be noted here is that the substitution in this side chain has very little effect in shifting the band towards the red end of the spectrum, although it has been shown that substitution in the ring by a carboxyl group shifts the band 100 A.U. in this sense. The alteration in the absorption follows the course which it would take if the compounds were entirely aliphatic. Henri has shown that the hydroxyl groups of aliphatic alcohols have an absorption band in the region of 2100 to 2200 A.U. This absorption band is

less intense than that given by corresponding aliphatic acid carboxyl groups. It is also further towards the extreme end of the ultra-violet. A similar relation holds between the lower parts of the curves of β-indole-ethyl alcohol and β-indolepropionic acid as can be seen in Fig. 1*a* and 1 *b*.

In the case of β-indole-aminopropionic acid there is both an acidic and a basic group, giving an opportunity for salt formation. Salt formation has been shown by Henri [1913] to shift the absorption band into the extreme end of the ultra-violet. Salt formation appears to have taken place in this case, since that part of the absorption spectrum corresponding to the amino and carboxyl groups is further in the ultra-violet than the corresponding part for β-indolepropionic acid. The intensity is also less.

The substitution of the side chain in the indole ring acts in a similar manner to the substitution of a methyl group and shifts the absorption band about 50 A.U. towards the red. But the intensity of the absorption is decreased from 9000 to 5000 units.

In examining group III it is seen that the two members are α- and β-di-substituted compounds, the α-substitution being in both cases the same, with the β-substitution in one case a hydroxyl group and in the other case an aldehyde group.

The most striking difference between this group and the previous groups is that instead of having only one main band as in groups I and II there are two well-marked bands, which occupy relatively the same positions to each other. The α-β-dihydroxyindole shows an absorption band in the region of 2200 and 2300 A.U. characteristic of the absorption band in aliphatic compounds having hydroxyl groups. The hydroxyindole-aldehyde also shows this absorption band but, as is to be expected, it is not so pronounced. The aldehyde group substituted in the ring shows the same effect in intensifying the absorption as it does in the case of indole-aldehyde. The shift in the bands towards the red of hydroxyindole-aldehyde compared with dihydroxyindole is about 500 A.U., whereas the shift in the band of indole-aldehyde compared with indole is about 400 A.U. The agreement in the effect of the aldehyde is quite good. The aldehyde absorption band does not show up so distinctly in hydroxyindole-aldehyde as in indole-aldehyde owing no doubt to the fact that the position is already occupied by one of the main bands of the parent substance.

In the case of group IV nothing much can be said owing to the lack of a number of compounds on which to make comparisons. In these two cases the double bond has disappeared from the pyrrole ring and the change in the absorption completely destroys any resemblance to the other three groups. These compounds are not indole compounds but indolin derivatives.

Sincere thanks are due to Professor Hopkins for his kindness in purchasing for the Biochemical Department the very specialised apparatus required for this research.

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CXII. THE ABSORPTION SPECTRA OF SOME AMINO ACIDS.

THE POSSIBLE RING STRUCTURE OF CYSTINE.

By FRED WILBERT WARD.
From the Biochemical Laboratory, Cambridge.

(*Received August 27th, 1923.*)

The object of this investigation was to find out whether the position of the absorption bands of amino acids bore any relation to the wave length of those ultra-violet rays which have proved most effective in the destruction of micro-organisms and for therapeutic purposes. When an organism is destroyed by the effects of radiation of any particular wave length a certain amount of work must have been done and a certain amount of energy corresponding to this work must have been utilised. The source of this energy is the rays absorbed by the tissues. In view of the fact that amino acids are the elements from which the protein compounds are built up and the fact that protein constitutes a large part of the non-aqueous part of protoplasm it would appear that there is a direct relationship between the absorption spectra of the amino acids and the effect of radiation on organised living matter.

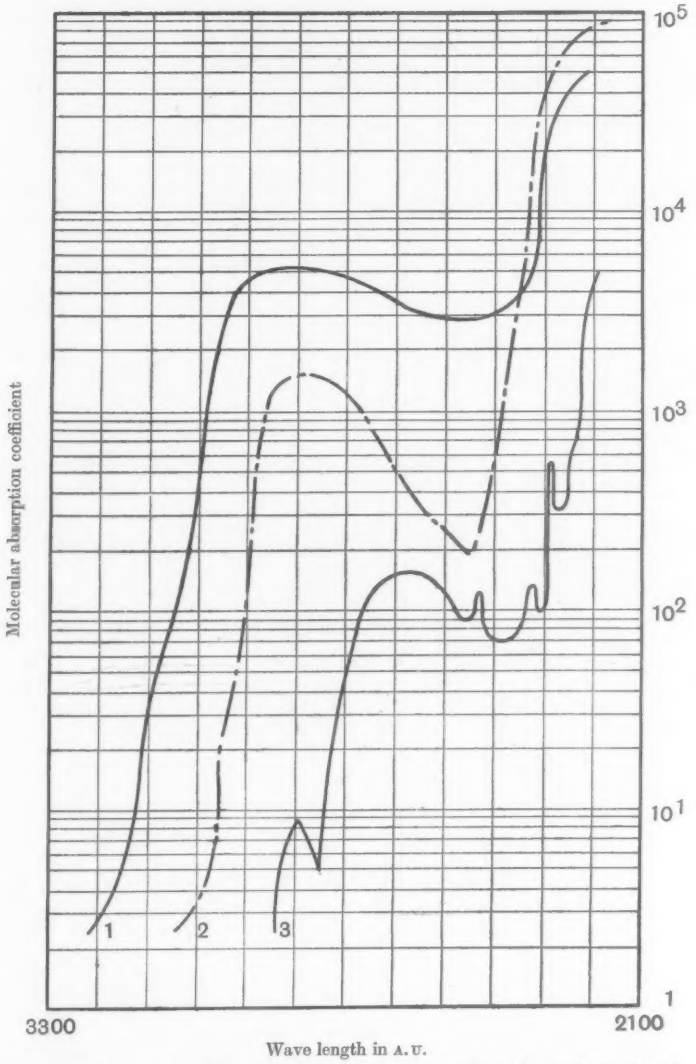
Experimental.

Method and Apparatus.

The method and apparatus employed in the study of the absorption spectra of these amino acids is the same as that employed by the present worker in the study of the absorption spectra of some indole compounds [Ward, 1923].

The absorption spectra of seven amino acids were measured. These were tryptophan, tyrosine, phenylalanine, alanine, histidine, glutamic acid and cystine. Of these seven amino acids, six were prepared in the Cambridge Biochemical laboratory. The remaining one, alanine, was purchased from British Drug Houses.

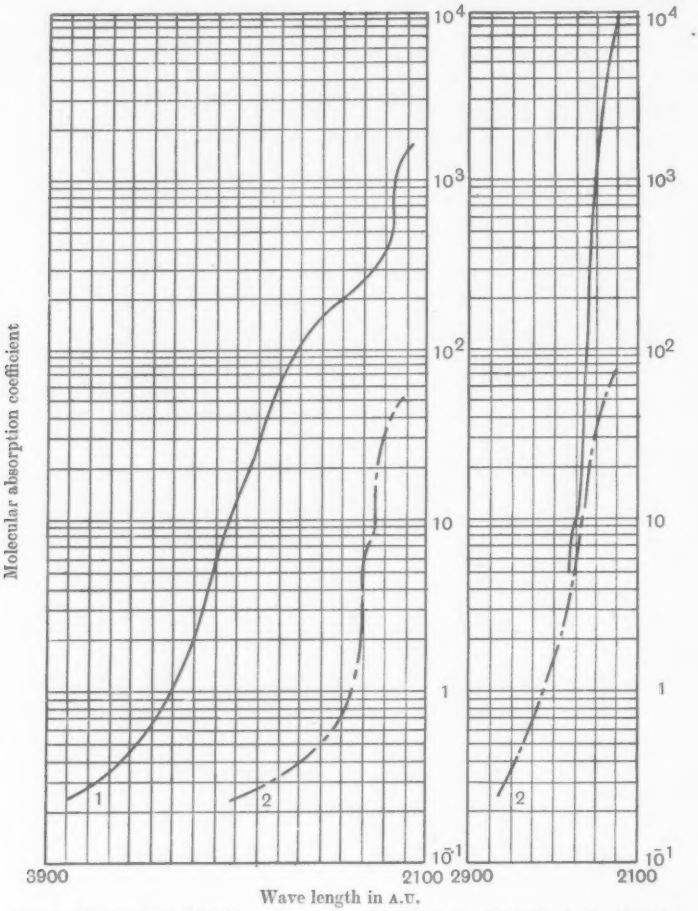
In making the absorption spectra measurements, solutions of a definite molecular concentration were used in order to obtain results that would have some basis of comparison. The solvent used for dissolving the amino acids was 50 % alcohol. Phenylalanine, tyrosine, histidine, glutamic acid and cystine were used in the form of the hydrochloride. Tryptophan and alanine were used in the form of the free acid. $M/100$ solutions of tryptophan, phenylalanine, tyrosine, and histidine were used as the strongest concentration and dilutions made from these solutions. Alanine and glutamic acid were used in $M/10$ concentration. Cystine was used in $M/20$ molar concentration and treated as if it were $M/10$ in plotting the results in order to obtain comparable results.



(1) Tryptophan. (2) Tyrosine hydrochloride. (3) Phenylalanine hydrochloride.

Fig. 1

The absorption spectra curves are plotted on Figs. 1 and 2. Fig. 1 gives the absorption spectra curves of tryptophan, tyrosine and phenylalanine, Fig. 2 *b* those of histidine and glutamic acid and Fig. 2 *a* those of cystine and alanine.



(*a*) (1) Cystine hydrochloride. (2) Alanine.

(*b*) (1) Histidine hydrochloride. (2) Glutamic hydrochloride.

Fig. 2.

Discussion.

Of the seven amino acids examined, the three acids containing the benzene ring—phenylalanine, tyrosine and tryptophan—give marked absorption bands. The others give only general absorption. Cystine gives a general absorption

with an intensity as great as that given by phenylalanine in low concentrations, and in high concentrations the absorption band extends to the edge of the visible spectrum. Kober [1915] has measured the edges of the bands of a number of amino acids without determining the complete absorption curve. From these results, it appears probable that the absorption spectra of the remaining amino acids have curves similar to those of glutamic acid and alanine.

The solar light contains ultra-violet rays that extend down to the wave length of about 3000 A.U. Cystine is the only amino acid apparently that has any marked absorption in the region of the solar ultra-violet light. It would appear suggestive that the presence of cystine in hair and wool where the concentration approaches that of about 10 %, is of physiological importance in the protection of the organism against the harmful effects of prolonged exposure to sunlight. The curious custom of the Arabs in wearing heavy but loosely fitting woollen clothing has apparently a strong justification in the protective effect of the cystine present in the wool.

Cystine appears to be present in high concentration only in the scleroproteins of hair and wool. Its concentration in the other classes of proteins is low and the absorption of ultra-violet rays would be of the same order as that caused by phenylalanine.

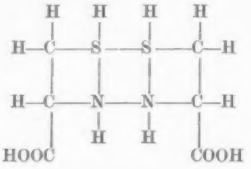
Only the ultra-violet light of the shorter wave lengths has great therapeutic value [Newcomer, 1917]. By shorter wave length is meant the rays of 2800 A.U. and shorter. These wave lengths have, however, very low penetrative power owing probably to the absorption by the outer tissues. The high absorption power of tryptophan, tyrosine and phenylalanine for the rays in the region of 2800 and 2900 A.U. throws some light on the effectiveness of these short wave lengths. These amino acids in low concentration have a high power of absorption and bring about a very complete absorption of these wave lengths. Consequently much energy is available for bringing about changes in the cell. If the amount of energy is so great that the chemical changes upset the normal metabolism of the cell, then the organism is destroyed.

The possible ring structure of cystine.

Cystine is soluble in strong mineral acids such as hydrochloric acid. It is insoluble in weak organic acids such as acetic acid. This is also characteristic of certain ring compounds containing an amino nitrogen as for example indole compounds. It is possible to make chloroacetyl derivatives of cystine by means of chloroacetyl chloride [Fisher and Suzuki, 1904], but apparently it is not possible to make acetyl derivatives of cystine by the use of acetyl chloride. Chloroacetic acid is a much stronger acid than acetic acid, being comparable in strength to hydrochloric acid.

The absorption of cystine is of a greater intensity than that of the aliphatic amino acids alanine and glutamic acid and is as strong as that of phenylalanine.

A possible explanation of these facts is that cystine does not possess the ordinary aliphatic structure usually assigned to it but that it possesses a ring structure, possibly of the following type:



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CXIII. THE ABSORPTION SPECTRA OF KYNURENIC ACID AND SOME RELATED QUINOLINE COMPOUNDS.

By FRED WILBERT WARD.

From the Biochemical Laboratory, Cambridge.

(*Received August 27th, 1923.*)

The absorption spectra of the following compounds were measured in the course of an investigation into the intermediary metabolism of indolepropionic acid [Ward, 1923, 2]. The injection of indolepropionic acid into the animal organism produces a body in the urine which is very unstable towards heat and is very difficult to isolate. It is the precursor of a bright cherry red pigment. The formation of kynurenic acid from tryptophan [Ellinger, 1904] and indolepyruvic acid [Ellinger and Matsuoka, 1920] suggested the possibility that a similar body might be formed from indolepropionic acid. The plan was to measure the absorption of the precursor of the pigment separated as completely as possible from other compounds found in the urine and to compare this absorption spectrum with the absorption spectra of known quinoline derivatives.

Experimental.

The apparatus and method employed were the same as that described by the present writer in a paper on the absorption spectra of some indole compounds [1923, 1].

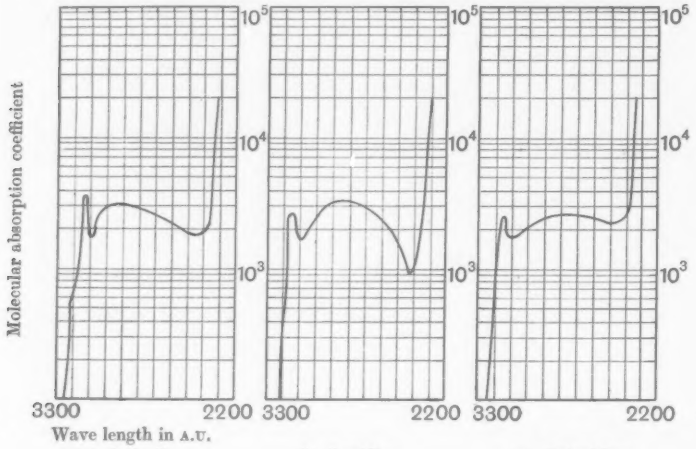
Seven different compounds of the quinoline series were investigated. These were as follows: quinoline, quinaldine, lepidine, quinaldinic acid, quinolylacetaldehyde, 2:6-dimethylquinoline and kynurenic acid.

The quinoline and quinaldine were purchased from British Drug Houses. The lepidine, quinaldinic acid, quinolylacetaldehyde and 2:6-dimethylquinoline were obtained from various sources in very small quantities of about two-tenths of a gram.

The kynurenic acid was prepared by feeding 20 g. of tryptophan to a dog at the rate of 4 g. per day. The kynurenic acid was first isolated in a crude state by acidifying the urine with hydrochloric acid and allowing to stand overnight. After filtering off the precipitated crude kynurenic acid and washing with water, it was dissolved in dilute ammonia and reprecipitated with dilute hydrochloric acid. By repeating this several times some of the urinary pigments were removed. It was then boiled up with 95 % alcohol and allowed to

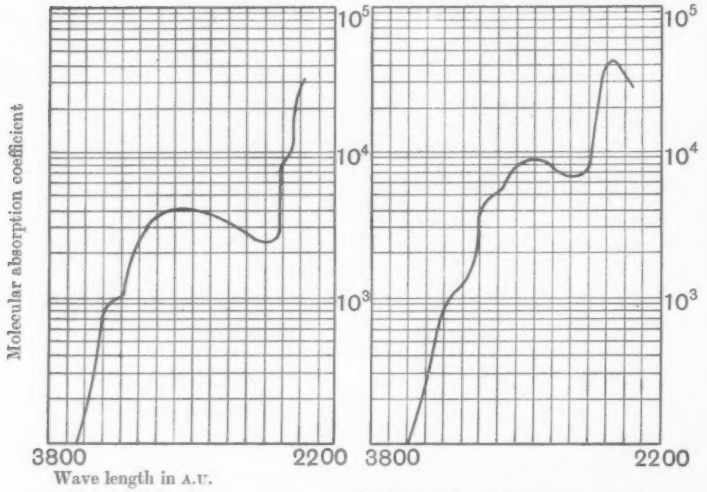
cool and then filtered. This removed some more of the pigment. It was finally recrystallised several times from 40 % acetic acid [Homer, 1914]. The final traces of pigment were removed by boiling in 40 % acid solution with animal charcoal before a final recrystallisation. This gave a white crystalline product.

The results are given in Figs. 1–3.



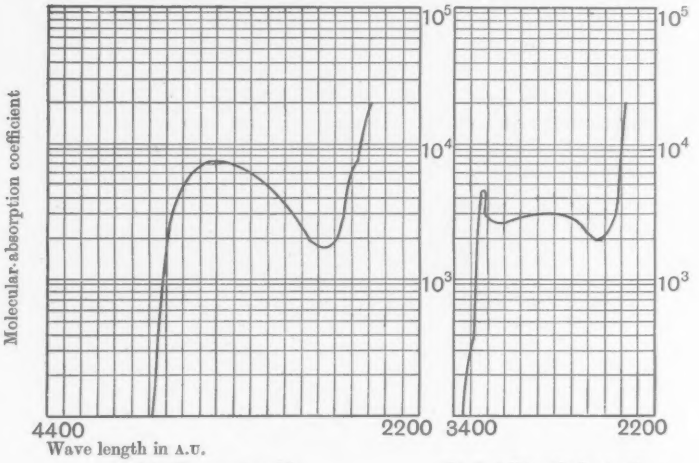
(*a*) Quinoline. (*b*) Lepidine. (*c*) Quinaldine.

Fig. 1.



(*a*) Quinaldinic acid. (*b*) Quinolylacetaldehyde (so-called).

Fig. 2.



(*a*) Kynurenic acid. (*b*) 2:6-Dimethylquinoline.

Fig. 3

Discussion.

From the absorption spectra of the three compounds quinoline, lepidine and quinaldine may be observed the difference of effect in the absorption spectra produced in one case by substituting in the α-position and in the other case in the γ-position. Substitution in the α-position produces a shift towards the red end of the spectrum without any increase in the intensity of the absorption. Substitution in the γ-position produces an increase in intensity of the absorption without producing any shift of the absorption band towards the red end of the spectrum.

Quinaldinic acid is quinoline substituted in the α-position by a carboxyl group. This brings about a well-marked change in the absorption as compared with quinaldine. There is a shift towards the red end of the spectrum of about 400 A.U. and there is also an increase in intensity.

Quinolylacetaldehyde is the name under which the compound whose absorption spectrum is given in Fig. 2 *b* is described in the literature [Einhorn, 1885, 1886; Carlier and Einhorn, 1890; Einhorn and Sherman, 1895]. It gives the correct elementary analysis for this constitution but it does not behave in its reactions as such a compound. It gives a hydrazone with great difficulty and in very poor yield. The absorption spectrum is not such as would be expected of such a compound.

2:6-Dimethylquinoline is a compound with a methyl group substituted in both rings. It retains a strong resemblance to quinaldine and quinoline. The effect of the double substitution is to increase the intensity of the absorption and to shift the absorption band towards the red end of the spectrum.

In kynurenic acid there is substitution in the α-position by a carboxyl group and in the γ-position by a hydroxyl group [Homer, 1913]. The presence of the carboxyl group in the α-position brings about a shift towards the red end of the spectrum with an increase in intensity. The presence of the hydroxyl in the γ-position brings about an increase in intensity. The effect of the two substitutions is to give a big shift towards the red end of the spectrum and greatly to intensify the absorption.

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CXIV. THE FATE OF INDOLEPROPIONIC ACID IN THE ANIMAL ORGANISM.

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(*Received August 27th, 1923.*)

Indolepropionic acid was first prepared by Nencki [1889] by the action of anaerobic bacteria upon commercial fibrin. He used three different strains of bacteria, namely *B. liquefaciens magnus*, *B. spinosus* and the Rauschbrand bacillus. He obtained the best yield of indolepropionic acid by the use of the Rauschbrand bacillus.

Hopkins and Cole [1903] subjected tryptophan to bacterial decomposition and obtained indole, indoleacetic acid and indolepropionic acid, proving that the amino acid was the precursor of the putrefactive decomposition bodies, indole, skatole, indoleacetic acid and indolepropionic acid. Indolepropionic acid was prepared by these workers by means of the Rauschbrand bacillus and *B. coli* working under anaerobic conditions in an atmosphere of hydrogen or nitrogen.

Ellinger [1904] fed tryptophan to a dog and found that there was an increased output of kynurenic acid in the urine. Some confusion existed for a long time as to the correct constitution of kynurenic acid, the two alternative forms being γ-hydroxy-α-carboxyquinoline and γ-hydroxy-β-carboxyquinoline. Homer [1913, 1914], by carefully recrystallising crude kynurenic acid from 40 % acetic acid, found that the melting-point agreed with that of γ-hydroxy-α-carboxyquinoline as prepared by Camps [1901].

Homer [1915] fed kynurenic acid to a dog but the material was excreted unchanged by the organism. It appears in that animal to be an end product of metabolism and not some intermediate product that has been excreted through being in excess.

Ellinger and Matsuoka [1914] prepared α-methyltryptophan and fed the product to a rabbit. They expected to obtain a methyl derivative of quinoline and to settle thereby in which form kynurenic acid exists. The only result of this experiment was that the α-methyltryptophan was recovered unchanged in the urine.

Barger and Ewins [1917] prepared α-methyltryptophan and fed it to a small dog. No kynurenic acid was found in the urine, the material being excreted in a form that left the indole ring intact.

Ellinger and Matsuoka [1920] prepared indolepyruvic acid and injected it into a rabbit. They obtained kynurenic acid in varying yields up to 11·7 %.

Injections of tryptophan gave yields of kynurenic acid up to 28 %. They also fed quinaldinic acid to rabbits but failed to obtain kynurenic acid as the oxidation product and found instead the quinaldinic acid conjugated with glycine.

Recently Matsuoka and Takemura [1922] have perfused the surviving liver of dogs and claim to have isolated kynurenic acid to the extent of 12 % after the addition of tryptophan and indolepyruvic acid to the perfusing fluid.

Homer [1915] made some spectrographic examinations of the pigment formed from indole compounds and from the urine of dogs fed with various indole compounds. She concluded that indole, skatole, indole-aldehyde, indole-carboxylic acid and indoleacetic acid undergo little change in the animal body but that indolepropionic acid appears to undergo a more deep-seated change in its passage through the animal organism.

Ewins and Laidlaw [1913] have shown that indole-ethylamine injected in the animal organism is excreted as indoleacetic acid conjugated with glycine. Similarly, when indoleacetic acid is injected into the animal organism, it is also excreted as a conjugate of indoleacetic acid and glycine. They have also shown that when indole-ethylamine is perfused through the surviving liver, the amine is oxidised to indoleacetic acid.

Guggenheim and Loeffler [1916] claim to have isolated indole-ethyl alcohol as a product of the perfusion of indole-ethylamine besides the previously isolated indoleacetic acid.

Experimental.

Preparation of Tryptophan and Indolepropionic acid.

The tryptophan required for the preparation of indolepropionic acid was made by the method of Hopkins and Cole [1901] with Onslow's modification [1921]. The product was finally extracted by butyl alcohol, according to Dakin's method [1918, 1920], a specially designed vacuum apparatus being used, in which the butyl alcohol boiled at about 65°.

Following the previous work of Hopkins and Cole [1903] several attempts were made to repeat the preparation of indolepropionic acid by means of pure cultures of *B. chauvei* obtained from the Lister Institute. Two such attempts failed and, as it was necessary to wait a month for the completion of an experiment, much time was lost. Finally twelve different strains of anaerobes were inoculated into small tubes of the media and kept under anaerobic conditions for a month in an incubator. These small tubes were tested after this lapse of time and four of them were found to have traces of ether-soluble derivatives of indole. These four were *B. coli*, *B. chauvei*, *B. sporogenes* and *B. oedemaciens*. Fresh twenty-four hour cultures were made of these bacteria and a five-litre bottle filled with sterilised medium containing 25 g. of tryptophan was inoculated with the whole four freshly growing cultures. The bottle was stoppered with a cork containing tubes for passing nitrogen gas through the bottle and with a mercury seal on the outlet. The air was removed by passing nitrogen through the culture and medium for a period of five hours.

When the bottle was opened at the end of four weeks, it was found to smell strongly of indole derivatives. In order to obtain the indolepropionic acid the solution was acidified with 5 % by volume of sulphuric acid and the indole compounds precipitated with mercuric sulphate-sulphuric acid solution. The precipitate was allowed to stand for twenty-four hours and then filtered off and washed. It was then suspended in warm water, made alkaline with baryta and the mercury compound decomposed with hydrogen sulphide. The solution was filtered from mercury sulphide and the residue washed. The filtrate and washings were united and acidified and extracted in the extraction apparatus with ether for four or five hours. The ether was then removed and the sticky mass of crystals was dissolved in alcohol, reduced in volume on a water-bath and water added. The indolepropionic acid was recrystallised from dilute alcohol after removing pigment by boiling with animal charcoal.

The average yield was about 30 % of the crude tryptophan used. In all, about 30 g. of indolepropionic acid were prepared.

Metabolism experiments with Indolepropionic acid.

Indolepropionic acid was dissolved in sodium bicarbonate solution and injected subcutaneously into a rabbit and the urine collected. When the urine was acidified with strong hydrochloric acid and boiled it developed a cherry-red colour. This colour is different from that obtained on injecting indoleacetic acid, acidifying the urine with hydrochloric acid, adding a drop of ferric chloride and boiling. It is also different from that obtained on heating free indoleacetic acid with hydrochloric acid.

3 g. were injected in the course of three days into four rabbits and the urine collected, care being taken to keep the collected urine in a cool place in presence of a preservative. The collected samples were precipitated with mercuric sulphate reagent by adding it until no further precipitate was obtained. The precipitate was filtered off and washed with distilled water. It was then suspended in distilled water, made alkaline with baryta, and decomposed with hydrogen sulphide. The mercury sulphide was filtered off and the baryta exactly removed by dilute sulphuric acid. The filtered solution was then evaporated to dryness on the water-bath. A red sticky gum was obtained which was dried in a vacuum desiccator over sulphuric acid. The red coloration developed as the evaporation proceeded and could not be prevented except in alkaline solution. It appeared to be a decomposition product of the precursor in the urine derived from the indolepropionic acid. Once the red pigment was formed, it acted very much like an indicator, turning yellow in alkaline solution and red in acid solution. Several attempts to purify this material by recrystallising were without success. The compound was further purified by dissolving in alkali, acidifying and extracting with butyl alcohol. It was then removed from the butyl alcohol by shaking with alkaline solutions of baryta, the baryta removed with dilute sulphuric acid and the solution again evaporated to dryness. The material was still tarry and could not be

got to crystallise from any of the ordinary solvents. This procedure was repeated several times but was not attended with any greater success.

Perfusion experiments were carried out with indolepropionic acid, tryptophan and indole-ethyl alcohol, but the only products that could be isolated were tarry materials soluble in alcohol but very resistant to all attempts at purification by recrystallisation. The liquid obtained at the end of a perfusion of tryptophan gave a positive nitroprusside test, as given by acetoacetic acid.

Absorption Spectra Experiments.

Owing to the difficulty of obtaining satisfactory results by the usual methods, it was evident that some other procedure must be used to obtain information as to the type of compound formed in the metabolism of indolepropionic acid. It was decided to attempt to make use of the quartz ultra-violet spectrophotometer in order to find out whether it would throw any light on the constitution of the compound present in the urine. There was the further inducement that it would be possible to record the absorption spectra of the compound without having to apply heat in its isolation.

In order to make use of this method it was first necessary to consider the possible metabolic products that might be obtained. This could be done by analogy with the known metabolism of similar compounds. Having listed the possible end products of the metabolism of indolepropionic acid, it was then necessary to make a study of these and related compounds as far as obtainable in order to observe the general shape of the absorption spectra curves and the effect of changing the substituting groups.

There appear to be four possible courses that the metabolism of indolepropionic acid could take which are as follows:

I. β-Oxidation of the side chain giving indolecarboxylic acid.

II. The oxidation of the carbon atom in the pyrrole ring giving rise to α-enol or α-keto indole derivatives.

III. A combination of types I and II giving rise to α-enol or α-keto β-carboxyindole.

IV. A splitting of the pyrrole ring and a linking up again with the formation of a quinoline derivative.

Following out this plan the absorption spectra of ten indole and seven quinoline compounds were measured. These divide themselves naturally into three groups.

Group I. *β-Substituted indole derivatives.*

(*a*) Tryptophan, indolepropionic acid and indole-ethyl alcohol.

(*b*) Indole, β-indolecarboxylic acid and β-indole-aldehyde.

Group II. *α- and β-Substituted indole derivatives.*

(*a*) $\alpha\beta$-Dihydroxyindole and α-hydroxy-β-indole-aldehyde.

(*b*) Isatin and sodium indigosulphonate.

Group III. *Quinoline compounds.*

Quinoline, quinaldine, lepidine, quinaldinic acid, quinolylacetaldehyde, 2:6-dimethylquinoline and kynurenic acid.

The spectra of the compounds of groups I and II have been described in a paper on indole compounds [1923, 1], and those of group III in a paper on kynurenic acid and some related quinoline compounds [1923, 2].

The absorption Spectra of the Metabolic Product of Indolepropionic acid.

Indolepropionic acid was injected subcutaneously into four rabbits and the urine collected over a period of twenty-four hours. The amount of indolepropionic acid used was 1 g. The urine was treated as described on p. 909, but the filtrate from the mercury sulphide was acidified and extracted with butyl alcohol. The butyl alcohol was then extracted with weak caustic soda solution and the separated aqueous layer was acidified and re-extracted with butyl alcohol. It was again extracted with dilute alkali and the alkaline solution acidified. This time the aqueous solution was extracted with ether and the ether extract washed with water. The aqueous solution was afterwards again extracted with butyl alcohol. In this manner two fractions were obtained, one soluble in butyl alcohol alone and the other soluble in butyl alcohol and in ether.

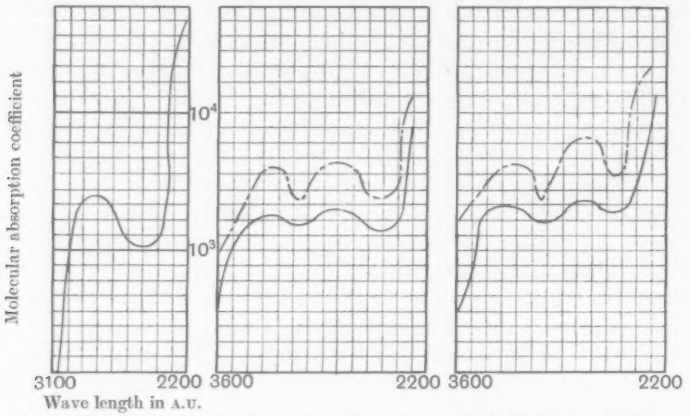
From these two fractions dilutions were made and the absorption spectra of the material measured. In these absorption spectra measurements there are two difficulties to be met with. The first of these is that the solutions are not pure and the second is that the concentration of the material in the solutions is unknown making it difficult to plot the results. However, it is possible to avoid these troubles to a certain extent. If the material to be determined is present in the solution in considerably greater concentration than the impurities, then these impurities will only interfere in the higher concentrations. As for the concentration certain corrections can be introduced based on the position of the peak of the bands and where they should appear in the correct concentrations.

The absorption spectra for indolepropionic acid and its metabolic product are given in Fig. 1. Fig. 1 *b* gives the absorption spectra of the ether-soluble fraction plotted in two ways, the lower uncorrected and the upper corrected for concentration. Fig. 1 *c* gives the absorption spectra curves of the alcohol-soluble fraction, the lower curve being uncorrected and the upper one corrected for concentration.

In order to show that this type of curve was due to some metabolic product of indolepropionic acid and not to impurities from the urine, two other compounds were also studied in the same way. These were indole-ethyl alcohol and indolecarboxylic acid.

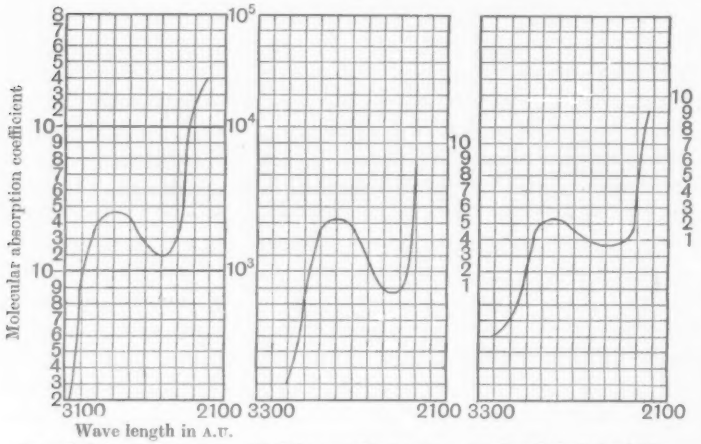
Indole-ethyl alcohol. 1 g. was dissolved in warm water and injected subcutaneously into four rabbits and the urine collected over a period of twenty-four hours. The urine was treated in exactly the same way as in the case of indolepropionic acid and the absorption spectra curves obtained of the two fractions. Further the ether-soluble fraction was identified as containing free indoleacetic acid. This was done in the following way. The ether solution was

treated with light petroleum to precipitate the pigments and tarry material. It was then allowed to stand and the clear solution decanted from the precipitated material. The mixed ethers were then evaporated on the water-bath. White waxy crystals were obtained which melted at 164°. The melting point of indoleacetic acid is given as 165°. Some of the material was dissolved in



(*a*) β-Indolepropionic acid. (*b*) Ether-soluble fraction. (*c*) Butyl alcohol-soluble fraction.

Fig. 1.

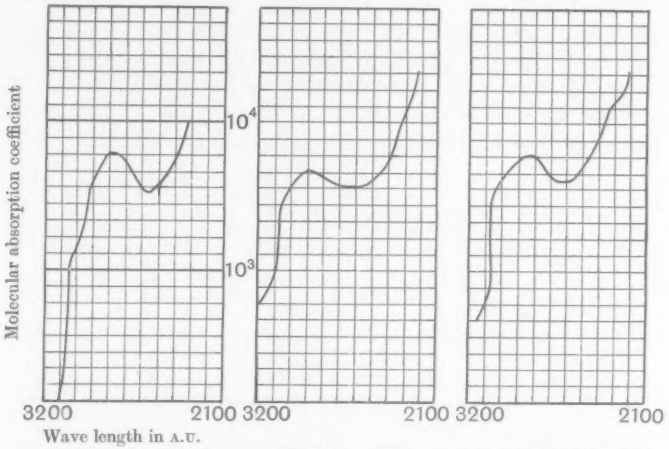


(*a*) β-Indole-ethyl alcohol. (*b*) Ether-soluble fraction. (Indoleacetic acid). (*c*) Butyl alcohol-soluble fraction.

Fig. 2.

water in a test-tube and boiled with strong hydrochloric acid. A red pigment was formed as given by free indoleacetic acid. The original urine also gave a red colour on boiling with strong hydrochloric acid and a drop of ferric chloride solution. The absorption spectra curves are shown in Fig. 2.

Indolecarboxylic acid. About a gram was injected into four rabbits and the urine collected over a period of twenty-four hours. The urine was treated as previously described for indolepropionic acid. Two fractions were again obtained and the absorption spectra measured. The urine on heating with hydrochloric acid gave a purplish colour but different from that given by indolepropionic acid. The absorption curves are given in Fig. 3.



(*a*) β-Indolecarboxylic acid. (*b*) Ether-soluble fraction. (*c*) Butyl alcohol-soluble fraction.

Fig. 3.

Discussion.

On examining the curves given for indole-ethyl alcohol and for indolecarboxylic acid, it can be seen that there has been no marked change in the absorption spectra given by the metabolic products from that given by the original substance. As can be seen by comparing them with the absorption spectra of β-substituted indole compounds they still retain the shape common to these.

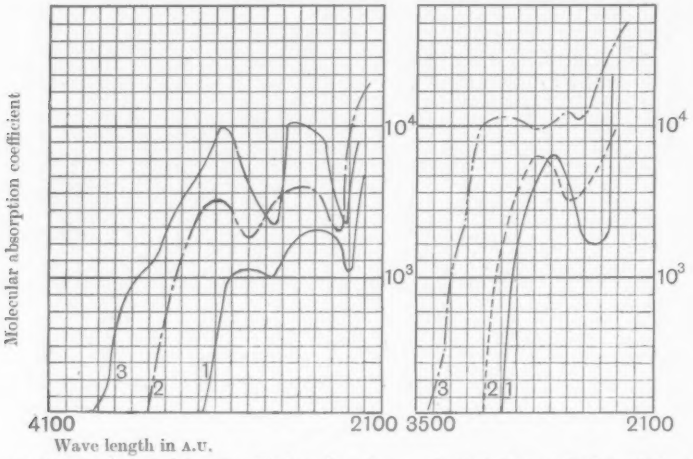
The absorption spectra of indolepropionic acid metabolic products show a marked difference from those of the original substance. The absorption spectra of these substances contain two wide bands, which is not characteristic of β-indole substituted compounds. On comparing the spectra with those given by quinoline compounds, no resemblance can be traced. However, on comparing the absorption spectra of the αβ-substituted compounds there is a noticeable resemblance.

In Fig. 4 the known absorption spectra curves of three β-substituted indole compounds and the absorption curves of two αβ-substituted compounds have been plotted. From these curves the absorption spectra curve of α-hydroxy-β-indolecarboxylic acid has been deduced and plotted. The resemblance

between the curve thus deduced and the curves of the metabolic products of indolepropionic acid is evident and strongly favours the suggestion of the formation of an α-hydroxyindole compound as the metabolic product of indolepropionic acid.

Attempts were made to prepare this compound from α-hydroxy-β-indolealdehyde by oxidation but it appeared to be very unstable as would be expected from the experience with the urinary compound. Several different oxidising reagents were employed but without success. It would appear necessary to stabilise the ring by methylating the hydroxyl group.

The fact that tryptophan and indolepyruvic acid yield kynurenic acid on being injected into the animal organism and the fact that α-methyltryptophan is excreted unchanged, suggest the possibility that the condition of the



(*a*) (1) Dihydroxyindole; (2) α-Hydroxy-β-indole-carboxylic acid (by analogy); (3) α-Hydroxy-β-indole-aldehyde.

(*b*) (1) Indole; (2) Indole-aldehyde; (3) Indolecarboxylic acid.

Fig. 4.

α-carbon atom in the pyrrole ring plays an important part in the metabolism of the indole ring as present in tryptophan. The presence of a methyl group would prevent oxidation at this point.

That the α-carbon atom is chemically reactive is shown from other sources. Hopkins and Cole [1903] oxidised tryptophan with ferric chloride and obtained two bases one of which was shown by Ellinger [1904] to be indole-aldehyde and the other was shown by Perkin and Robinson [1919] to be identical with harman, a derivative of harmine. Perkin and Robinson have prepared this substance by oxidising tryptophan in the presence of acetaldehyde. Under this treatment the end of the aliphatic side chain joined on to the α-carbon atom.

SUMMARY.

Indolepropionic acid in its passage through the animal body appears to undergo oxidation in the α-carbon atom of the pyrrole ring with formation of an α-hydroxyindole compound, which may be in either the enol or keto form.

Indole-ethyl alcohol in its passage through the animal organism undergoes oxidation to indoleacetic acid.

Indolecarboxylic acid, in its passage through the animal body, appears to be unchanged except that it is excreted in a conjugated form. It is not an intermediate step in the metabolism of indolepropionic acid.

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CXV. COLLOIDS AND HAEMOLYSIS.

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In the investigation of haemolytic phenomena the majority of experimenters have made use of washed corpuscles, which are more or less completely divested of the colloids of the plasma. During life, however, the erythrocytes of the blood are invariably associated with colloidal solutions. If, then, we are to gain any insight into the nature of haemolysis in the living organism, it appears essential to study the action of haemolytic substances also in association with colloids.

That serum exerts a protective action in haemolysis has been demonstrated by a number of observers. Moore, Wilson and Hutchinson [1909], Kerner [1910] and Sutherland and Mitra [1916] all recorded the inhibitory action of serum on haemolysis by sodium oleate, while Barker [1922] has noted the differences in the inhibitory power against haemolysis by this substance afforded by sera obtained from human blood during various forms of anaemia. Kuno [1918] found that the lysis produced by heating human erythrocytes was inhibited by the presence of human serum. Fenn [1922] stated that 0·1 % of serum inhibits the haemolysis arising from the contact of erythrocytes with soiled glass. Ponder [1921, 1922, 1923] observed that serum inhibits the haemolytic action of urine and affords protection to human erythrocytes against haemolysis by a histamine-glycocholate mixture, by sodium taurocholate, by sodium glycocholate and by saponin.

Schmidt and Norman [1920] described the inhibition of the haemolysis produced by eosin in the presence of sunlight by serum, egg-white, caseinogen, edestin and "peptone" and stated that gelatin failed to restrain this photodynamic action. Pickering and Collins [1923] pointed out the protective action of isotonic gum-saline against the haemolysis of human erythrocytes at 13° by X-rays.

The objects of the present investigation are as follows: to ascertain the variations in the resistance to haemolysis of erythrocytes obtained from different individuals of the same species, both human and animal, and the effect of storage thereon: to enquire into the influence on haemolysis of various colloids, viz. gum acacia, gelatin, egg-white and sera, fresh and "inactivated": to investigate the influence of varying concentrations of saponin and of a naturally haemolytic serum on the speed and titre of haemolysis in the presence

and absence of the colloids named and to enquire into possible modifications in the protection afforded by these colloids by alterations in the hydrion concentration in the media employed.

Eisler [1909] found that sugar inhibits specific haemolysis, that the addition of moderate amounts of various salts restores lysis, higher concentrations of the electrolytes again inducing inhibition. Purdy and Walbum [1922] found that certain metallic salts hasten haemolysis by saponin at some concentrations and show opposite effects at other concentrations. Periodicity or a so-called zone phenomenon has been described by Buchanan [1919] in the agglutination of meningococci by cerous nitrate, while Holker [1923] has demonstrated the occurrence of periodicity in the influence of increasing concentrations of sodium chloride on the opacity of human serum. Early in this investigation the influence of saponin dissolved in isotonic saline on haemolysis was found to be often periodic in nature. The effect of colloidal solutions on this periodicity was therefore studied.

The Technique and Terms Employed.

The tests for haemolysis were carried out in Dreyer's tubes both in the water-bath at 38°, controlled by a thermostat, and at room temperatures, which were read and noted at the time of each experiment. Haemolysis in the water-bath took place in the absence of light (except at the actual times of reading). The experiments at room temperatures were in the presence of diffuse daylight. The possible influence of daylight and of ultra-violet light on haemolysis by saponin and by the heterologous serum employed will be enquired into later. By "commencement" of haemolysis we mean the first change in opacity visible to the naked eye by transmitted light, by "completion" when the contents of the tube were free from residue, when similarly observed. In the tables "H. begins" indicates the time of "commencement" of haemolysis; "H. ends" shows the time of "completion" of that process.

The blood corpuscles employed were those of the sheep, guinea-pig, white rat and man. Except in the case of whole blood they were obtained by bleeding the animals into sterile glass tubes, defibrinating with glass beads, thrice washing in normal saline and centrifuging. 25 % suspensions were made up in sterile tubes in 0·86 % NaCl, dissolved in distilled water. The haemolysing agents used were saponin (Merck) and the serum of a rabbit which was haemolytic at 38° to all the erythrocytes used, except those of the rabbit. Variations in the p_H of the protective colloids were made by rendering them neutral, faintly alkaline and faintly acid to litmus, by the addition of small quantities of $N/100$ HCl and $N/100$ NaOH respectively. The protective action in each case was then tested. Control experiments showed that the colloidal substances so treated were devoid of haemolytic action.

In each experiment the bulk of the haemolytic substance together with the protective colloid employed and the normal saline used for dilution was 1 cc. to which 0·004 cc. of a 25 % suspension of the various corpuscles was added.

Complete haemolysis is indicated by +, when more than half of the suspension is haemolysed ± is used, when less than half ∓ is recorded. The letters N.C.H. imply nearly complete haemolysis. Each suspension undergoing haemolysis was compared with a standard suspension showing no haemolysis after centrifuging or settling out.

Variations in the Resistance of Erythrocytes to Haemolysis.

Considerable variations occur in the titre and speeds of haemolysis in individuals of the same species, but erythrocytes withdrawn under the same conditions on consecutive days from the same animal exhibit but little alteration in sensitiveness to haemolysis by saponin and by the haemolytic serum used. With human whole blood individual variations in sensitiveness to saponin are well marked, as shown in Table I. The temperature of haemolysis was 13°. Two readings (marked A and B) of the commencement and completion of haemolysis are shown for each concentration of saponin on corpuscles from bleedings from the same person taken at an interval of one week. In each case the reading B was with blood withdrawn one week later than that used for reading A. Each bleeding was made in the afternoon.

Table I. *Showing Individual Variations in the Sensitiveness of Human Blood to Haemolysis by Saponin.*

Concentrations of saponin in 0·86 % NaCl		1/10,000 A	1/10,000 B	1/20,000 A	1/20,000 B
1. age 22 ♂	H. begins	2′ 0″	2′ 10″	3′ 30″	3′ 30″
	H. ends	3 30	3 30	10 30	10 15
2. age 23 ♂	H. begins	2 30	2 30	trace 1 hr.	– 1 hr.
	H. ends	6 0	5 55	+24 hrs.	+24 hrs.
3. age 26 ♂	H. begins	2 30	2 25	± 1 hr.	± 1 hr.
	H. ends	3 20	3 30	+24 hrs.	+24 hrs.
4. age 29 ♂	H. begins	1 10	2 0	– 1 hr.	– 1 hr.
	H. ends	4 0	4 0	trace 24 hrs.	trace 24 hrs.
5. age 30 ♂	H. begins	0 55	1 0	– 1 hr.	trace 1 hr.
	H. ends	1 30	1 35	±24 hrs.	±24 hrs.
6. age 31 ♂	H. begins	1 40	1 45	– 1 hr.	– 1 hr.
	H. ends	3 0	3 0	N.C.H. 24 hrs.	N.C.H. 24 hrs.
7. age 55 ♂	H. begins	1 0	1 0	– 1 hr.	– 1 hr.
	H. ends	4 0	3 35	±24 hrs.	±24 hrs.
8. age 26 ♀	H. begins	3 30	3 30	– 1 hr.	–24 hrs.
	H. ends	4 0	4 5	trace 24 hrs.	
9. age 28 ♀	H. begins	2 0	2 10	– 1 hr.	∓ 1 hr.
	H. ends	4 30	4 30	trace 24 hrs.	∓24 hrs.
10. age 35 ♀	H. begins	2 30	2 30	– 1 hr.	– 1 hr.
	H. ends	4 0	4 10	trace 24 hrs.	∓24 hrs.
11. age 48 ♀	H. begins	1 0	1 10	– 1 hr.	–24 hrs.
	H. ends	1 30	1 30	trace 24 hrs.	
12. age 54 ♀	H. begins	2 0	2 30	– 1 hr.	– 1 hr.
	H. ends	3 10	3 10	+24 hrs.	±24 hrs.

The following may be noted:

(1) The speeds of haemolysis by saponin of whole blood taken from the same person at the interval of one week are remarkably constant.

(2) The relatively rapid haemolysis of whole blood by a high concentration of saponin does not imply a relatively low resistance to lower concentrations of saponin. Thus the corpuscles of one individual (No. 11) which were completely haemolysed in 1′ 30″ by 1/10,000 saponin, exhibited incomplete haemolysis after exposure for 24 hours to 1/20,000 saponin, whereas the corpuscles of another individual (No. 1) which were haemolysed in 3′ 30″ by 1/10,000 saponin, exhibited complete haemolysis after exposure for 10′ 30″ to 1/20,000 saponin. In an isolated case (blood from a male aged 25), 1/40,000 of saponin gave partial haemolysis in 2 hours and complete haemolysis in 24 hours.

(3) Similar variations in individual sensitiveness to haemolysis by saponin occur with the thrice washed corpuscles of man, the guinea-pig, white rat, sheep and rabbit, greater variations being found in the speed than in the titre of haemolysis of the corpuscles examined; those of the sheep appear to exhibit the least individual variations in sensitiveness to haemolysis by saponin and, in this connection, the high resistance exhibited by the erythrocytes of this animal to haemolysis by saponin may be recalled. The thrice washed corpuscles of the guinea-pig, which are hypersensitive to saponin, exhibit as marked individual variations in sensitiveness to haemolysis by that substance as does the whole blood of that animal or the whole blood or washed erythrocytes of man.

It was found that the resistance of sheep's corpuscles to saponin, as shown by the titre and speed of haemolysis, underwent little alteration when the erythrocytes were kept for two days in sterile tubes at room temperatures (13°–14°) or for three days in an ice-chest. After these periods resistance is definitely lower as is shown by the next table.

Table II. *Showing Deterioration in Resistance to Haemolysis by Saponin due to Storage.*

Concentrations of saponin dissolved in 0·86 % NaCl		1/800	1/1600	1/3200	1/6400	1/12800	1/25600
Fresh erythrocytes (sheep)	H. begins	3′ 50″	9′ 30″	21′ 30″	– 24 hrs.	– 24 hrs.	– 24 hrs.
	H. ends	4 40	18 30	39 30			
Erythrocytes kept 4 days in ice-chest (sheep)	H. begins	2 0	2 30	8 0	12′ 0″	18′ 0″	– 1 hr.
	H. ends	3 0	4 15	14 0	28 0	32 0	±24 hrs.

A similar lowering of resistance to haemolysis by saponin was observed when the erythrocytes of the guinea-pig, white rat and rabbit were kept, under the conditions named, for similar periods.

Table III. *Showing Deterioration in Resistance to Haemolysis by a Heterologous Serum due to Storage.*

Percentages of rabbit's serum employed		25 %	12·5 %	6·25 %	3·125 %
Fresh erythrocytes (sheep)	H. begins	2′ 0″	16′ 0″	– 2 hrs.	– 24 hrs.
	H. ends	4 0	18 0	±24 hrs.	
Erythrocytes kept 4 days at 13° (sheep)	H. begins	at once	9 0	21′ 0″	– 24 hrs.
	H. ends	„	15 0	42 0	

Similar results were obtained with fresh and "four day" guinea-pig's corpuscles, when the haemolysing agent was serum from the same rabbit.

The Protective Action of Gum Acacia.

A 7 % solution of gum acacia, dissolved in 0·86 % NaCl, was used. One half of the fluid of the suspensions was of this material, the remainder being saponin, in the dilutions named, dissolved in isotonic saline. In the control experiments the suspensions were in 0·86 % NaCl. The protective action of gum-saline against the haemolysis at 13° by saponin of fresh sheep's corpuscles is usually very slight and may be absent. When the corpuscles have remained in sterile tubes in an ice-chest, excluded from daylight, the protective action of gum acacia is well marked at 13° against certain concentrations of saponin, as is shown in Table IV.

Table IV. *Showing the Protection afforded by Gum-saline against the Haemolysis of Stored Erythrocytes by Saponin.*

Concentrations of saponin used		1/800	1/1600	1/3200	1/12800	1/25600	1/51200
Sheep's erythrocytes suspended in 0·86 % NaCl	H. begins	2′ 0″	2′ 30″	8′ 0″	18′ 0″	– 1 hr.	– 24 hrs.
	H. ends	3 0	4 15	14 0	33 0	±24 hrs.	trace 48 hrs.
Sheep's erythrocytes suspended in gum-saline	H. begins	2 0	5 50	8 0	– 24 hrs.	– 24 hrs.	– 48 hrs.
	H. ends	3 0	12 0	17 30			

Table V records the speeds of haemolysis of the fresh erythrocytes of the sheep at 38°, and of the corpuscles of the guinea-pig, white rat and rabbit, at room temperatures, by saponin dissolved in 0·86 % NaCl and by like concentrations of saponin in the presence of 50 % of a 7 % solution of gum acacia dissolved in 0·86 % NaCl. The commercial gum acacia employed was faintly acid to litmus and contained salts of calcium.

Hitherto the appearance of periodicity, as illustrated by changes in the agglutination of erythrocytes, in the opacity of serum and in specific haemolysis, has been associated with variations in the concentration of the electrolytes present. Reference to Table V will show that periodicity occurs in the haemolysis of sheep's corpuscles at 38° and at lower temperatures with the erythrocytes of the guinea-pig and white rat, when the suspensions are made and the saponin is dissolved in normal saline. In these cases the variant is the concentration of the saponin and not the total amount of electrolyte present.

That temperature may also be a factor in periodicity in haemolysis is illustrated by the fact that the phenomenon is frequently absent in the haemolysis of sheep's corpuscles by saponin at 13° but is well marked at 38°.

It appears probable that periodicity in haemolysis, under the conditions named, is due to alterations in the local concentrations of electrolytes at the interfaces of the corpuscles by the varying concentrations of saponin present. If this is so, physical phenomena are involved in the inception of haemolysis by saponin.

With the erythrocytes of a guinea-pig suspended in normal saline the presence of 1/51,200 of saponin caused relatively slow haemolysis accompanied by marked sedimentation. The presence of 1/102,400 of saponin gave more rapid haemolysis and sedimentation was absent. The corpuscles of another guinea-pig exhibited slow haemolysis accompanied by sedimentation when

Table V. *Showing the Action of Gum-saline and the Occurrence of Periodicity in the Haemolysis of Fresh Erythrocytes by Saponin.*

Concentrations of saponin used		1/6400	1/12800	1/25600	1/51200	1/102400
Sheep's erythrocytes in 0·86 % NaCl at 38°	H. begins	complete	2′ 0″	8′ 0″	30′ 0″	– 48 hrs.
	H. ends	in 60″	3 0	∓ 2 hrs. ±24 hrs.	∓24 hrs.	
Sheep's erythrocytes in 50 % gum-saline at 38°	H. begins	complete	2 30	21′ 0″	– 120′	– 48 hrs.
	H. ends	in 1′ 50″	7 30	±2 hrs. N.C.H. 24 hrs.	slight 24 hrs.	
Guinea-pig's corpuscles in 0·86 % NaCl at 13°	H. begins	0′ 50″	4 30	7′ 30″	6′ 30″	12′ 45″
	H. ends	1 30	8 30	13 0	11 30	15 40
Guinea-pig's corpuscles in 50 % gum-saline at 13°	H. begins	0 50	5 50	10 0	10 0	– 24 hrs.
	H. ends	1 30	8 30	– 2 hrs. ±24 hrs.	– 120′ ±24 hrs.	
White rat's corpuscles in 0·86 % NaCl at 13°	H. begins	0 30	1 30	1′ 30″	3′ 55″	27′ 0″
	H. ends	1 20	2 20	2 30	28 0	±120 0 +24 hrs.
White rat's corpuscles in 50 % gum-saline at 13°	H. begins	0 30	4 0	12 15	– 48 hrs.	– 24 hrs.
	H. ends	1 30	55 0	∓24 hrs.		∓48 hrs.
Rabbit's corpuscles in 0·86 % NaCl at 14°	H. begins	at once	1′ 0	2 0	3′ 50″	7′ 0″
	H. ends		1 40	3 0	13 0	14 0
Rabbit's corpuscles in 50 % gum-saline at 14°	H. begins	1′ 50″	4 30	10 20	∓30′ 0″	– 24 hrs.
	H. ends	2 10	5 0	12 30	+4 hrs.	

Concentrations of saponin used		1/204800	1/409600	1/819200	1/1638400	1/3276800
Sheep's erythrocytes in 0·86 % NaCl at 38°	H. begins	2′ 30″	2′ 0″	54′ 0″	– 24 hrs.	– 24 hrs.
	H. ends	3 30	3 30	±24 hrs.		
Sheep's erythrocytes in 50 % gum-saline at 38°	H. begins	16 0	3 0	3′ 50″	– 120′ 0″	trace
	H. ends	N.C.H. 24 hrs.	8 0	9 0	trace 24 hrs.	24 hrs.
Guinea-pig's corpuscles in 0·86 % NaCl at 13°	H. begins	3′ 30″	1 20	4 45	40′ 0″	∓24 hrs.
	H. ends	3 55	3 20	8 35	N.C.H. 24 hrs.	
Guinea-pig's corpuscles in 50 % gum-saline at 13°	H. begins	– 2 hrs.	– 2 hrs.	– 24 hrs.	– 24 hrs.	– 24 hrs.
	H. ends	∓24 hrs.	∓24 hrs.			
White rat's corpuscles in 0·86 % NaCl at 13°	H. begins	28′ 0″	7′ 0″	– 60′ 0″	– 60′ 0″	46′ 0″
	H. ends	±24 hrs.	N.C.H. 24 hrs.	±24 hrs.	±24 hrs.	∓60 0 +24 hrs.
White rat's corpuscles in 50 % gum-saline at 13°	H. begins	– 24 hrs.	– 24 hrs.	– 48 hrs.	– 48 hrs.	– 48 hrs
	H. ends	+48 hrs.	+48 hrs.			
Rabbit's corpuscles in 0·86 % NaCl at 14°	H. begins	– 48 hrs.	– 48 hrs.	,,	,,	,,
	H. ends					
Rabbit's corpuscles in 50 % gum-saline at 14°	H. begins	,,	,,	,,	,,	,,
	H. ends					

1/102,400 of saponin was present. With 1/204,800 of saponin haemolysis was very rapid and sedimentation was absent. With 1/1,638,400 haemolysis was very slow but sedimentation was absent. Sedimentation of the corpuscles of the guinea-pig was also observed in the presence of gum with concentrations of saponin varying from 1/51,200 to 1/102,400 with corpuscles from different animals, the sedimentation always corresponding with relatively slow haemolysis. With higher titres of saponin sedimentation appeared slight or absent. Sedimentation was not observed when gelatin or egg-white was substituted for gum.

The fresh corpuscles of the sheep at 38°, in the presence of gum-saline, exhibit periodic inhibition of haemolysis by saponin at different concentra-

tions of the latter substance than when the gum is absent. For example, the presence of gum afforded marked protection against 1/25,600 and 1/51,200 of saponin and protection was also afforded against 1/204,800 of saponin, yet with 1/819,200 of that substance haemolysis was slow with the erythrocytes suspended in normal saline but very rapid with those (from the same animal) which were suspended in gum-saline. With 1/102,400 of saponin inhibition of haemolysis occurred with both saline and gum suspensions. A zone phenomenon may be marked in the haemolysis of the corpuscles of the guinea-pig at 13° by saponin dissolved in normal saline, but may be only slightly evident when corpuscles from the same animal are suspended in gum-saline. With the corpuscles of the white rat periodicity in haemolysis by saponin was found to be almost absent in presence of gum (see Table V). In the case of the rabbit it was not found either with protected or unprotected corpuscles, and it was noted that the protection afforded by gum against haemolysis by saponin is greater with the corpuscles of the rabbit than with those of the white rat, guinea-pig or sheep.

That individual variations occur in the appearance of periodicity in the haemolytic action of saponin is shown by Table VI.

Table VI.

Individual Variations in the Periodicity in Haemolysis by Saponin.

Animal type	Temp. of haemolysis	Condition of erythrocytes	Proportion of animals with corpuscles which exhibited periodicity
Sheep	13°	fresh in 0·86 % NaCl	1 out of 5
,,	13°	3rd day in 0·86 % NaCl	3 ,, 5
,,	13° and 38°	fresh in gum-saline	each 10 ,, 10
,,	38°	3rd day in gum-saline	14 ,, 15
Rabbit	13° and 38°	fresh and 3rd day in 0·86 % NaCl	each 0 ,, 5
,,	13° ,, 38°	,, ,, gum-saline	,, 0 ,, 5
Guinea-pig	13° ,, 16°	fresh in 0·86 % NaCl	2 ,, 12
,,	38°	,, ,,	4 ,, 5
,,	13°	,, gum-saline	1 ,, 5
,,	38°	,, ,,	3 ,, 5
White rat	13°	,, 0·86 % NaCl	3 ,, 6
,,	13°	,, gum-saline	0 ,, 6

Gum saline also protects the fresh erythrocytes of the guinea-pig and sheep against the haemolytic action of fresh rabbit's serum at 38°. (The serum employed was also haemolytic to the corpuscles of the sheep at 38°.)

Table VII. *Showing the Protective Action of Gum-saline against Haemolysis by a Heterologous Serum.*

Proportions of rabbit's serum present		1/2	1/4	1/8	1/16	1/32
Unprotected guinea-pig's corpuscles	H. begins	at once	2′ 0″	3′ 30″	−24 hrs.	−24 hrs.
	H. ends		3 0	∓2 hrs. +24 hrs.		
Guinea-pig's corpuscles protected by gum	H. begins	1′ 50″	56 0	−24 hrs.	−24 ,,	−24 ,,
	H. ends	7 0	123 0			
Unprotected sheep's corpuscles	H. begins	at once	2 0	6′ 30″	− 2 ,,	− 2 ,,
	H. ends		4 0	8 30	±24 ,,	±24 ,,
Sheep's corpuscles protected by gum	H. begins	at once	4 0	120 0	− 2 ,,	−24 ,,
	H. ends		9 0	148 0	±24 ,,	−24 ,,

The Influence on Haemolysis of Alteration in the Hydrion Concentration of Saponin Solutions.

Isotonic solutions of saponin were prepared which were respectively neutral, faintly alkaline and faintly acid to litmus. Table VIII illustrates the speed and titre of haemolysis of the erythrocytes of the guinea-pig and rabbit when exposed to these solutions at a temperature of 14°.

Table VIII. *Showing the Titre and Speed of Haemolysis by Saponin in Neutral, faintly Alkaline and Acid Solutions.*

Concentrations of saponin used		1/12800	1/25600	1/51200	1/102400	1/204800	1/409600
Guinea-pig's corpuscles in neutral saponin	H. begins	2′ 0″	2′ 30″	11′ 0″	– 2 hrs.	22′ 0″	– 2 hrs.
	H. ends	5 30	5 30	57 0		∓2 hrs.	
Guinea-pig's corpuscles in alkaline saponin	H. begins	at once	0 30	2 0	16′ 30″	22′ 0″	,,
	H. ends		0 50	5 0	±2 hrs.		
Guinea-pig's corpuscles in acid saponin	H. begins	at once	0 30	2 10	3′ 40″	11 0	,,
	H. ends		0 55	3 45	6 50	∓2 hrs	
Guinea-pig's corpuscles in neutral saponin	H. begins	0′ 40″	2 40	9 0	– 2 hrs.	– 2 hrs.	– 2 hrs.
	H. ends	1 30	7 50	18 0			
Guinea-pig's corpuscles in alkaline saponin	H. begins	0 10	0 40	1 10	8′ 30″	,,	,,
	H. ends	0 30	1 50	2 10	24 30		
Guinea-pig's corpuscles in acid saponin	H. begins	0 30	0 40	2 50	– 2 hrs.	,,	,,
	H. ends	0 45	1 30	4 50			
Rabbit's corpuscles in neutral saponin	H. begins	0 50	1 50	2 10	7′ 0″	,,	,,
	H. ends	1 10	3 10	6 50	20 0		
Rabbit's corpuscles in alkaline saponin	H. begins	at once	0 10	0 35	4 40	20′ 30″	30′ 0″
	H. ends		0 40	1 0	17 40	49 0	∓2 hrs.
Rabbit's corpuscles in acid saponin	H. begins	0′ 40″	1 10	1 30	5 50	17 0	– 2 hrs.
	H. ends	0 45	1 40	2 20	18 0	trace 2 hrs.	

Table VIII shows that faintly alkaline and faintly acid saponin are more haemolytic at 14° than is neutral saponin. Like results were obtained when the temperature was 38°. Periodicity in haemolysis by saponin was not observed when the saponin was either faintly alkaline or faintly acid to litmus, even with corpuscles which exhibited periodicity in haemolysis by neutral saponin.

The Effects of Altering the Hydrion Concentration of the Protective Colloids.

Table IX shows the variations in the protection afforded by isotonic gelatin against the haemolysis of fresh erythrocytes of the guinea-pig by saponin at 14° when the added gelatin is made neutral, faintly alkaline and faintly acid to litmus by the addition of hydrochloric acid and sodium hydroxide respectively.

The substitution of gum-saline for isotonic gelatin yields similar results; the gum which is faintly acid to litmus being more protective than gum which is faintly alkaline or neutral.

Table IX. *Showing the Protection against Saponin afforded by Neutral, Alkaline and Acid Gelatin.*

Concentrations of saponin used		1/12800	1/25600	1/51200	1/102400	1/204800	1/409600	1/819200	1/1638400
Unprotected erythrocytes	H. begins	1′ 50″	4′ 50″	6′ 40″	53′ 0″	2′ 30″	1′ 30″	5′ 45″	40′ 0″
	H. ends	2 55	12 30	12 10	100 0	3 10	2 30	9 10	∓ 2 hrs.
Erythrocytes in 50 % neutral gelatin	H. begins	3 0	32 0	68 0	96 0			∓24 hrs.	
	H. ends	7 0	47 0	110 0	122 0	−48 hrs.	−48 hrs.	+48 hrs.	−48 hrs.
Erythrocytes in 50 % alkaline gelatin	H. begins	2 40	18 0	30 0	66 0				
	H. ends	7 10	36 0	47 0	120 0	,,	,,	−48 hrs.	,,
Erythrocytes in 50 % acid gelatin	H. begins	1 50	8 0	−24 hrs.					
	H. ends	5 0	∓ 2 hrs. +24 hrs.	∓48 hrs.	−48 hrs.	,,	,,	,,	,,

In Table X is shown the protective power of neutral alkaline and acid gum against haemolysis of guinea-pig's erythrocytes by rabbit's serum at 38°.

Table X. *Showing the Protection against Haemolysis by a Heterologous Serum afforded by Neutral, Alkaline and Acid Gum.*

Proportions of rabbit's serum employed		1/2	1/4	1/8	1/16	1/32
Unprotected erythrocytes	H. begins	at once	1′ 30″	2′ 50″	30′ 0″	− 2 hrs,
	H. ends	,,	4 20	26 0	∓ 2 hrs. + 4 ,,	+ 4 ,,
Erythrocytes in 50 % neutral gum	H. begins	1′ 30″	16 0	50 0	100′ 0″	−24 ,,
	H. ends	6 0	32 0	∓2 hrs. +24 ,,	±24 hrs.	
Erythrocytes in 50 % alkaline gum	H. begins	4 30	18 0	60′ 0″	116′ 30″	−24 ,,
	H. ends	26 0	34 0	± 2 hrs. +24 ,,	±24 hrs.	
Erythrocytes in 50 % acid gum	H. begins	1 50	56 0	−24 ,,	−24 ,,	−24 ,,
	H. ends	7 0	123 0			

Table XI illustrates the variations in the protective power of 50 % of egg-white, when untreated by reagents and respectively faintly alkaline and faintly acid to litmus, against the haemolysis of the corpuscles of the guinea-pig by saponin at 14°.

Table XI. *Showing the Protection afforded against Saponin by Natural, Alkaline and Acid Egg-white.*

Concentrations of saponin used		1/6400	1/12800	1/25600	1/51200	1/102400
Unprotected erythrocytes	H. begins	0′ 50″	2′ 40″	4′ 30″	8′ 30″	2′ 45″
	H. ends	1 30	4 30	7 30	12 50	5 45
Erythrocytes in untreated egg-white	H. begins	0 50	2 30	60 0	−24 hrs.	−24 hrs.
	H. ends	1 30	4 50	±24 hrs.		
Erythrocytes in alkaline egg-white	H. begins	1 0	2 50	−24 hrs.	,,	,,
	H. ends	1 30	5 0			
Erythrocytes in acid egg-white	H. begins	at once	1 0	2′ 30″	6′ 0″	−24 hrs.
	H. ends		1 30	8 0	60 0	

Concentrations of saponin used		1/204800	1/409600	1/819200	1/1638400
Unprotected erythrocytes	H. begins	2′ 35″	1′ 20″	4′ 45″	40′ 0″
	H. ends	3 0	2 20	8 30	N.C.H. 24 hrs.
Erythrocytes in untreated egg-white	H. begins	−24 hrs.	−24 hrs.	−24 hrs.	−24 hrs.
	H. ends				
Erythrocytes in alkaline egg-white	H. begins	,,	,,	,,	,,
	H. ends				
Erythrocytes in acid egg-white	H. begins	,,	,,	,,	,,
	H. ends				

It will be noted that the protection afforded against haemolysis by saponin is greatest when the egg-white is in faintly alkaline solution and is least in faintly acid solution, untreated solutions occupying an intermediate position. The protection afforded by acid egg-white is however considerable. Periodicity in the action of the saponin was exhibited by the unprotected corpuscles, but appeared absent when egg-white was present. The contrast with the behaviour of gelatin in acid and alkaline solutions should be noted (*vide* Table IX). In this connection, it is noteworthy that Bayliss [1906] found that egg-white protects Congo red against the action of electrolytes in alkaline solution but exerts an opposite effect in acid solution.

When the haemolytic agency is the serum of the rabbit (at 38°) and the protective colloid is 50 % of egg-white the results with guinea-pig's corpuscles are different.

Table XII. *Showing Protection by Natural, Alkaline and Acid Egg-white against Haemolysis by a Heterologous Serum.*

Proportions of rabbit's serum used		1/2	1/4	1/8	1/16
Unprotected erythrocytes	H. begins	at once	1′ 30″	1′ 50″	30′ 0″
	H. ends	,,	4 20	26 0	∓120 0 +24 hrs.
Erythrocytes in natural egg-white	H. begins	12′ 0″	−1 hr.	− 1 hr.	− 1 hr.
	H. ends	60 0	N.C.H. 24 hrs.	∓24 hrs.	±24 hrs.
Erythrocytes in alkaline egg-white	H. begins	1 10	4′ 50″	7′ 0″	∓ 1 hr.
	H. ends	6 0	32 0	49 30	±24 hrs.
Erythrocytes in acid egg-white	H. begins	1 30	2 55	6 30	∓ 1 hr.
	H. ends	11 0	135 0	90 0	±24 hrs.

Similar results to those recorded in Tables XI and XII were found when white rat's corpuscles were substituted for those of the guinea-pig. The erythrocytes of the rabbit exposed to saponin in the presence of acid, alkaline and untreated egg-white and gelatin respectively gave results similar to, but less marked than, those obtained with the corpuscles of the guinea-pig. A serum haemolytic to rabbit's corpuscles was not available.

Table XIII. *Showing Protection by Fresh and "Inactivated" Rabbit's Serum against Haemolysis by Saponin.*

Concentrations of saponin used		1/400	1/800	1/1600	1/3200	1/6400	1/12800
Unprotected sheep's corpuscles	H. begins	1′ 30″	4′ 0″	6′ 30″	11′ 0″	20′ 0″	45′ 0″
	H. ends	2 30	6 30	9 30	14 10	27 30	±24 hrs.
Corpuscles protected by fresh serum	H. begins	8 0	14 0	−1 hr.	−24 hrs.	−24 hrs.	−24 hrs.
	H. ends	12 0	16 0	N.C.H. 24 hrs.			
Corpuscles protected by serum "inactivated" by storage	H. begins	14 0	−1 hr.	−24 hrs.	,,	,,	,,
	H. ends	48 0	trace 24 hrs.				
Corpuscles protected by serum "inactivated" by heating to 56°	H. begins	13 0	trace	,,	,,	,,	,,
	H. ends	48 0	1 hr. trace 24 hrs.				

The Protective Action of Certain Sera.

Table XIII shows the protection afforded by 50 % of rabbit's serum, when fresh and "inactivated," by heating to 56° and by storage, against the haemo-

lysis of sheep's corpuscles at 11·5° by saponin. The fresh serum employed was haemolytic to sheep's corpuscles at 38°.

Table XIV shows the protection afforded by 50 % of rabbit's serum, when fresh and "inactivated," either by heating to 56° or by storage, against the haemolysis of rabbit's corpuscles at 14° by saponin.

Table XIV. *Showing Protection by Fresh and "Inactivated" Rabbit's Serum against Haemolysis by Saponin.*

Concentrations of saponin used		1/3200	1/6400	1/12800	1/25600	1/51200	1/102400
Unprotected rabbit's erythrocytes	H. begins	at once	at once	1′ 0″	1′ 5″	2′ 50″	6′ 40″
	H. ends	,,	,,	1 40	1 40	3 0	13 0
Rabbit's erythrocytes in fresh serum	H. begins	11′ 30″	∓24 hrs.	− 48 hrs.	− 48 hrs.	− 48 hrs.	− 48 hrs.
	H. ends	14 0	+48 hrs.				
Rabbit's erythrocytes in serum "inactivated" by storage	H. begins	− 48 hrs.	− 48 hrs.	,,	,,	,,	,,
	H. ends						
Rabbit's erythrocytes in serum "inactivated" by heating	H. begins	− 24 hrs.					
	H. ends	∓48 hrs.	,,	,,	,,	,,	,,

The following may be noted concerning the protection afforded by sera against the haemolysis of washed erythrocytes by saponin.

(1) A fresh serum, which is haemolytic at 38°, may be protective at room temperatures (13°–14°) against haemolysis by saponin. Thus a serum may be protective at a low temperature and haemolytic at body temperature.

(2) The sera of the rabbit, guinea-pig and white rat, when "inactivated" either by heating to 56° or by storage, are protective against the haemolysis by saponin of rabbit's, guinea-pig's and white rat's corpuscles at room and body temperatures. The "inactivated" sera exhibit rather greater protective qualities than do the fresh sera.

(3) Rabbit's serum, fresh and "inactivated" either by storage or heating (obtained from an animal haemolytic to the sheep), protects rabbit's corpuscles against haemolysis by saponin at 13° and at 38°. A like protection is afforded by the serum (fresh and "inactivated") of the white rat to the corpuscles of the white rat at 13° and at 38°. Horse serum, when "inactivated" by storage, is powerfully protective against the haemolysis by saponin at 13° and at 38° of the corpuscles of the sheep, guinea-pig and white rat.

Table XV shows the protective power of 50 % horse serum ("inactivated" by storage) against the haemolytic action of fresh rabbit's serum at 38° on the corpuscles of the guinea-pig and the sheep.

Table XV. *Showing Protection by "Inactivated" Horse Serum against Haemolysis by Rabbit's Serum.*

Proportion of rabbit's serum used		1/2	1/4	1/8	1/16
Unprotected corpuscles of guinea-pig	H. begins	at once	2′ 0″	3′ 30″	46′ 30″
	H. ends	,,	3 0	∓24 hrs.	∓24 hrs.
Guinea-pig's corpuscles in horse serum	H. begins	2′ 0″	trace 24 hrs.	− 24 hrs.	− 24 hrs.
	H. ends	23 0			
Unprotected corpuscles of the sheep	H. begins	at once	2′ 0″	6′ 30″	− 120′ 0″
	H. ends	,,	4 30	8 30	± 24 hrs.
Sheep's corpuscles in horse serum	H. begins	60′ 0″	− 24 hrs.	− 24 hrs.	− 24 hrs.
	H. ends	N.C.H. 2 hrs. +24 hrs.			

Preliminary experiments with fresh serum of the rabbit and with the "inactivated" serum of the horse indicate that their respective protective qualities are modified by rendering the media of the suspensions faintly acid and faintly alkaline to litmus. It is proposed, however, to defer the publication of this work until more experiments have been conducted.

Summary and Conclusion.

(1) Individual variations occur in the sensitiveness to haemolysis by saponin in whole human blood and in the washed erythrocytes of man, the guinea-pig, white rat, sheep and rabbit.

(2) The individual variations in sensitiveness described are due to variations in the condition of the erythrocytes and not to differences in any plasma or serum which may be adherent to them.

(3) Relatively rapid haemolysis by a high concentration of saponin does not imply a relatively low resistance to lower concentrations of saponin.

(4) The resistance of the erythrocytes of the sheep, guinea-pig, white rat and rabbit to haemolysis by saponin is lowered by keeping the corpuscles in normal saline for longer than three days in sterile tubes at either room temperature or that of an ice-chest.

(5) Periodicity is exhibited in the haemolysis of sheep's corpuscles at 38° by saponin dissolved in 0·86 % NaCl. A similar periodicity occurs in the haemolysis at room temperatures of the corpuscles of the guinea-pig and white rat by saponin dissolved in normal saline.

(6) The occurrence of periodicity in haemolysis by saponin may be modified by alterations in the temperature of the corpuscles.

(7) The presence of gum acacia alters the concentrations at which periodic inhibition of haemolysis occurs in the action of saponin on the erythrocytes of the sheep. Periodicity in haemolysis by saponin appears to be absent when the corpuscles of the white rat and guinea-pig are suspended in gum-saline.

(8) Individual variations occur in the appearance or absence of periodicity in haemolysis by saponin. Periodicity in saponin haemolysis appears to be absent when rabbit's erythrocytes are used.

(9) The association of sedimentation of corpuscles with the exhibition of periodicity in haemolysis is described.

(10) Isotonic gum-saline, isotonic gelatin, egg-white, the "inactivated" serum of the horse, the fresh serum of the rabbit and that "inactivated" either by storage or by heating to 56° protect in varying degrees the erythrocytes of the sheep, white rat, rabbit, and guinea-pig against haemolysis by saponin at room temperatures (13°–14°).

(11) The serum of a rabbit, which at 38° was haemolytic to the corpuscles of the sheep, protects the erythrocytes of the latter animal against haemolysis by saponin at 13° to 14°. The same serum may thus, at different temperatures, be haemolytic and protective respectively.

(12) "Inactivated" serum is more highly protective than is the fresh serum of the same animal.

(13) Gum-saline, isotonic gelatin, egg-white and "inactivated" horse serum protect the erythrocytes of the guinea-pig against the haemolytic action of the serum of the rabbit at body temperature.

(14) The protective action of gum acacia, gelatin, egg-white and of certain sera against haemolysis by saponin or by a haemolytic serum is modified by altering the hydrion concentration of the respective colloids. Faintly acid egg-white is *less* protective against saponin than is faintly alkaline egg-white. Faintly acid egg-white is *more* protective against the haemolysis of guinea-pig's corpuscles by fresh rabbit's serum than is slightly alkaline egg-white.

(15) In view of the results here recorded, it appears premature to accept any of the current hypotheses concerning haemolysis either by saponin or by haemolytic sera. Indications are, however, forthcoming that physical processes play a part in the inception of haemolysis by saponin.

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